METHODS AND COMPOSITIONS FOR TREATING CANCER

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BACKGROUND OF THE INVENTION

The exposure of humans and other mammals to heavy metals ranges can generate useful, therapeutic anti-cancer or anti-inflammatory responses to severe toxic and poisoning reactions (Suzuki-Kurasaki *et al* 1997 *J. Histochem. Cytochem, 45*:1493-1501). Unfortunately, the differences in heavy metal concentration that may result in a therapeutic reaction versus an adverse reaction are very small. The mammalian protein, metallothionein is produced by several tissues and circulates throughout the bloodstream. It mediates renal exposure and toxicity to heavy metals by complexing with the heavy metals *in vivo*. Metals that complex with metallothionein include metals encountered during environmental and occupational exposure, such as cadmium and mercury; metals involved in genetic disorders, e.g., copper; as well as metals used for therapeutic purposes, e.g., gold and platinum. Thus, metallothionein metabolism is critical in the fields of human physiology and therapeutics.

For example, environmental and occupational exposure to cadmium is widespread, but mostly chronic and low-level. Whether ingested or inhaled, the majority of absorbed cadmium eventually complexes with metallothionein (Nordberg, M. 1984 *Environ. Health Perspect., 54*:13-20; Chan, HM *et al*, 1993 *Toxicol. Appl. Pharacol., 123*:89-96). The resulting Cd metallothionein complex is small enough (~7 kDa) to be freely filtered through the renal glomerulus into the proximal tubular fluid before reuptake into proximal tubular cells (Foulkes, EC, "*Role of metallothionein in epithelial transport and sequestration of cadmium*" in Metallothionein in Biology and Medicine, Klassen CD, Suzuki KT (eds), RC Press, Boca Raton, FL, 1991:pp171-182). Although neither the metallothionein apoprotein nor the zinc complex appears toxic, the heavy metal complex Cd-metallothionein is a renal tubular toxin. Damage

caused by such a toxin includes proteinuria, glucosuria, and aminoaciduria, or in more severe cases, acute tubular necrosis or chronic renal failure (Rehm S, Waalkes MP, **1990** *Toxicol. Appl. Pharmacol. 104*:94-105).

Heavy metals when used therapeutically are also limited by their renal toxicity. Metallothionein carries the commonly prescribed therapeutic heavy metals, gold and cisplatinum, throughout the body. Gold therapy is predominantly used to treat rheumatoid arthritis, but the duration and dosage of gold administration are limited by nephrotoxicity (Saito, S and Kurasaki, M., **1996** Res. *Commun. Mol. Pathol. Pharmacol., 93*:101-107; Saito, S and Kojima, Y, **1996** *Commun. Mol. Pathol. Pharmacol., 92*: 119-126; Glennas, A. et al, **1986** *Biochem. Pharmacol., 35*:2033-40). Cisplatinum, one of the most commonly prescribed chemotherapeutic agents, contains the heavy metal platinum. It has activity against diverse solid tumors including prostate, bladder, head and neck, ovarian, and lung cancers. However, cisplatin therapy is dosed to avoid acute dose-dependent nephrotoxicity in patients. More clinically significant is the limitation of the duration of cisplatin therapy due to cumulative nephrotoxicity. Both of these therapeutic heavy metals are limited in acute dose selection and duration of chronic therapy by nephrotoxicity based in the renal tubules (Ramesh, G. and Reeves, WB. **2002** *J. Clin. Invest., 100*:835-42; Boogaard, PJ *et al*, **1990** *Contrib. Nephrol., 83*:208-12).

Genetic mutations of metallothionein, leading to reduced copper binding, are also implicated in several copper storage diseases (Suzuki-Kurasaki et al, cited above; Okabe, M. et al, "*Relationship between Cu metabolism hereditary disorders and distribution of Cu metallothionein in kidneys*" in Metallothionein IV, Klaassen, CD (ed), 1999 Birkhauser Verlag, Basel, GE pp413-419).

In all of these cases, the major site of damage caused by exposure to heavy metals is the proximal tubules of the kidney. Free cadmium, copper and cisplatinum are toxic predominantly in the S_3 proximal straight tubule (Sabolic, I. *et al*, **2002** *Am. J. Physiol. Renal.*, 283:F1389-F1402; Ramesh and Reeves, cited above; Okabe et al, cited above). In contrast, conjugation of the heavy metals to metallothionein appears to shift the damage to S_1 and S_2 sub-segments in the renal cortex (Young, IT., **1977** *J. Histochem., Cytochem. 25*:935-941; Okabe et al, cited above).

The different roles of intracellular and circulating MT have created much confusion about the role of MT in cytotoxicity (Liu J. et al, 1994 *Toxicol Appl Pharmacol* 128: 264–270; Nordberg M. 1984 *Environ Health Perspect* 54: 13–20; Ramesh G and Reeves WB. 2000 *J Clin Invest* 110: 835–842). Several lines of evidence suggest that increased intracellular MT is a scavenger for heavy metals, providing protection against the effects of free heavy metals (Foulkes EC, "Role of metallothionein in epithelial transport and sequestration of cadmium." In: *Metallothionein in Biology and Medicine*, ed. Klaassen CD and Suzuki KT., Boca Raton, FL: CRC, 1991, p. 171–182; Fowler BA et al, "Proximal tubule cell injury." In: *Metallothionein in Biology and Medicine*, ed. Klaassen CD and Suzuki KT. Boca Raton, FL: CRC, 1991, p. 311–321; and Nordberg, cited above). This is one basis for the practice of administering bismuth to induce tissue MT clinically, before administration of the heavy metal-based chemotherapeutic agent cisplatinum.

To design appropriate protective strategies against the heavy metal-caused nephrotoxicity, the pathway by which metallothionein enters proximal tubular cells must be identified. Conjugation of heavy metals, such as copper or cadmium, to metallothionein changes the nephron site of renal uptake of the heavy metals and also greatly enhances the nephrotoxic effect of these agents (Okabe M, et al, "Relationship between Cu metabolism hereditary disorders and distribution of Cu metallothionein in kidneys." In: *Metallothionein IV*, ed. Klaassen CD. Basel: Birkha˜user Verlag, 1999, p. 413–419; Sabolic I, et al, 2002. *Am J Physiol Renal Physiol* 283: F1389–F1402). Changes in the nephron site of uptake of heavy metals follow conjugation to metallothionein, when free Cu is taken up in the inner medulla of mice. However, preconjugation of the Cu to metallothionein changed the uptake to the cortex of the kidney (Okabe et al, cited above). A similar observation was made with cadmium, including morphological analysis of the nephron segments from S_3 for un-conjugated cadmium to peri-glomerular S_1 for metallothionein conjugated cadmium. Metallothionein conjugation of cadmium was found to increase the tubular toxicity enormously (Sabolic et al, cited above).

The entry route of heavy metal-metallothionein complexes into the epithelial cells remains unknown (Nordberg and Nordberg, 1987 *J. UOEH. 9* Suppl.:153-164;

Fowler, *et al*, "*Proximal Tubule Cell Injury*" in Metallothionein in Biology and Medicine, Klassen, CD, Suzuki KT (eds)l, CRC Press, Boca Raton, FL, 1991: pp311-321; Liu *et al*, **2001** *Toxicol. Appl. Pharmacol., 175*:253-259; Endo *et al*, **2000** *Toxicol.*, 146: 187-95; Kroning, *et al*, **1999** *Br. J. Cancer, 79*:293-299; Sharma and McQueen, **1982** *Biochem. Pharmacol., 31*:2153-9; Kone et al, **1990** *J. Membr. Biol., 113*:1-12). Conflicting reports implicate different transporters or receptor mediated pathways (Foulkes, cited above; Bernard *et al*, **1988** *Kidney Int., 34*:185-85; Bernard *et al*, **1988** *Toxicol. Appl. Pharmacol., 87*:440-5; Tsuruoka *et al*, **2000** *J. Pharmacol. Exp. Ther., 292*:769-77; Kinne *et al*, **1995** *Toxicol., Appl. Pharmacol, 1356*:216-21; Marshall *et al*, **1994** *J. Submicrosc. Cytol. Pathol., 26*:59-66). At least some of the uncertainty arises from the use of *in vivo* and *in vi*tro models that differ significantly in their behavior. For example, while $CdCl_2$ is more toxic than Cd-metallothionein to cultured rat kidney proximal tubules and LLC-PK1 cells, Cd-metallothionein shows greater *in vivo* nephrotoxic effects (Liu *et al*, **1994** *Toxicol. Appl. Pharmacol., 128*:264-70; Prozialeck *et al*, **1993** *Life Sci., 53*:PL337-42; and Sabolic *et al*, cited above). The lack of consensus complicates the search for a therapy for renal heavy metal poisoning regardless of the source of the metal.

There is a clear need in the art for an appropriate therapy that permits urinary excretion of cadmium, secondary to inhibition of renal proximal tubular uptake of Cd or Cu metallothionein in the treatment of cadmium and copper storage diseases, and to eliminate the toxic accumulation of this heavy metal from environmental sources. There is also a need in the therapeutic uses of heavy metals for inhibition of renal uptake of the metals to enable the broadening of dose selection and treatment duration thereof.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a pharmaceutical composition useful for the treatment of cancer which includes a conjugate formed by a modified metallothionein (MT) amino acid sequence or fragment thereof that binds the megalin receptor less avidly than naturally-occurring metallothionein and multiple molecules

of a therapeutic divalent metal ion. In one embodiment the modified MT does not bind megalin.

In another aspect, the invention comprises a composition formed by the described conjugate which further includes a delivery peptide for targeted delivery to a desired cell, wherein said delivery peptide is fused to the modified MT or fragment thereof.

In another aspect, the invention includes a method for treating cancer comprising administering to a mammalian subject an effective amount of the pharmaceutical composition described herein. This method inhibits the renal uptake of divalent metal ions.

Another aspect of this invention is the use of the above-defined compositions in the preparation of a medicament for the treatment of cancer.

Still another aspect of this invention includes a method for inhibiting renal uptake of therapeutic divalent metals ions by administering the metal ions in admixture with a conjugate of the above-defined composition.

In yet a further aspect, the invention provides a method of manufacturing an above-defined conjugate by synthesizing a modified MT amino acid sequence or fragment thereof that does not bind megalin with zinc divalent ions complexed thereto; lowering the pH of the synthesized product to remove zinc therefrom; dialyzing the synthesized product and contacting it with the selected therapeutic divalent metal ion; and either raising the pH to complex the selected metal ion to the sequence or incubating with the selected metal.

Yet a further aspect of this invention includes a metallothionein derivative amino acid sequence or fragment thereof that does not bind megalin as avidly as naturally occurring metallothionein, the sequence optionally binding metal ions. These sequences are not taken up by cells of the kidney, including proximal tubular cells, cells of the ear or inner ear, or other cells of the body. In one embodiment, such metallothionein derivatives are mutated at the highly conserved hinge or interdomain region, centered on a lysine repeat, so that such derivatives bind heavy metals but do not bind megalin and thus are not taken up by proximal tubular cells.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the sequence of metallothionein class I (MT) SEQ ID NO: 1, indicating cadmium (Cd) binding sites and α and β domains below the sequence. Aligned peptide fragments of SEQ ID NO: 1 used in interference studies assayed by surface plasmon resonance (SPR) techniques are indicated above the MT sequence. The lysine repeat that appears in the hinge region is indicated.

FIG. 2 indicates the results of surface plasmon resistance (SPR) analysis of the dose-dependent binding of rabbit kidney MT in HEPES-buffered saline (HBS) containing 2 mM Ca and Mg to megalin. A graph plotting resonance units (RU) vs. MT molar concentration shows fit of the maximum responses obtained after 2.5 minutes. The double-referencing method of (Myszka DG. 1999 *J Mol Recognit* 12: 279–284) was used to eliminate artifacts in the data.

FIG. 3 is a bar graph indicating the flow cytometry analysis of the displacement of fluorescently labeled MT from brush-border membranes by anti-receptor antisera. Brush-border membrane vesicles isolated from rat renal cortex were incubated with fluorescently conjugated MT and receptor antisera. The observed fluorescence is shown for the control (MT alone) and MT in the presence of anti-cubilin, anti-megalin, and anti-NK1-peptide antibodies. Data files of 2,000 observations/sample were collected.

FIG. 4 is a bar graph of the flow cytometry analysis employed to show the displacement of fluorescently labeled MT from brush-border membranes by peptides and ligands. Values are means ± SE. Brush-border membrane vesicles isolated from rat renal cortex were incubated with fluorescently conjugated MT and various peptides and ligands. The observed fluorescence is shown for the control (no MT), and fluorescent MT alone (MT-no competition), as well as the effect of added *peptide 2* (amino acids 10–26 of SEQ ID NO: 1), overlap peptide SCKKSCC (amino acids 28–34 of SEQ ID NO: 1), known megalin ligand β2-microglobulin, and antibodies to the AT1 receptor (AT1R; a control for nonspecific binding), and competition with

equimolar concentration of unlabeled MT (MT-competition). Data files of 2,000 observations/sample were collected; $n = 6$.

FIG. 5A is a graph showing the flow cytometry analysis of the dose dependence of fluorescently labeled MT into BN-16 cells. Concentration of MT producing half-maximal uptake is 4–8 μM. In view of this data, all later experiments used at least 4 μM MT (10 μM preferred when reagents were not limiting).

FIG. 5B is a graph showing the flow cytometry analysis of the time dependence of fluorescently labeled MT into BN-16 cells. Nearly linear uptake of MT-FLUORX was observed over 3+ h at 2 doses. As a result, uptake experiments with antibodies and MT-Cy3 were performed for 1–2h.

FIG. 5C is a graph showing competitive uptake of fluorescently labeled MT into BN-16 cells. Known megalin ligand β2-microglobulin displayed dose-dependent interference with MT uptake over a broad range of concentrations of both β2-microglobulin and MT.

FIG. 6A is a bar graph showing flow cytometry analysis of antibody inhibition of uptake of fluorescently labeled MT into BN-16 cells. Anti-cubilin antisera inhibited MT uptake into BN-16 cells in a concentration-dependent manner. The effect of anti-megalin (meg) antiserum was far greater than anti-cubilin (cub) antiserum; the 2 sera produced an additive effect. Anti-AT1R antiserum, which also binds BN-16 cells, was used as a nonspecific binding control but had no effect on MT uptake.

FIG. 6B is a bar graph showing flow cytometry analysis of peptide inhibition of uptake of fluorescently labeled MT into BN-16 cells. *Peptide 4*, containing the overlap sequence SCKKSCC (amino acids 28-34 of SEQ ID NO: 1) inhibited MT uptake, as did the overlap sequence itself. In contrast, *peptide 2*, distant from the overlap sequence but with heavy cysteine content, did not affect MT uptake. Concentrations of all peptides were 100 μM.

FIG. 6C is a bar graph showing flow cytometry analysis of recombinant (recomb) protein inhibition of uptake of fluorescently labeled MT into BN-16 cells. Recombinant (recomb) full-length mouse MT inhibited the uptake of fluorescently labeled MT by BN-16 cells, as did the α-subunit carrying the intact SCKKSCC

(amino acids 28-34 of SEQ ID NO: 1) motif. The β-subunit, in which this motif is disrupted, was far less effective at inhibiting MT uptake.

FIG. 7 is a bar graph indicating the effects on renal function in mice treated with a control, with cisplatin alone (cisplat), with a complex formed of MT and zinc (ZnMT), with a complex formed of MT and cisplatin (MT-cis), a complex of this invention formed of the MT-α subunit only with cisplatin (alpha-cis) and a complex of this invention formed of the MT-β subunit with cisplatin (beta-cis) as discussed in Example 8 below.

FIG. 8 is a bar graph illustrating the results of a 4 hour uptake into DMS53 small cell lung cancer cells of phosphate buffered saline (PBS), cisplatin alone (cis), metallothionein alone (MT), a complex formed of MT and cisplatin (MT-cis), a complex of this invention formed of the MT-α subunit only with cisplatin (alpha-cis) and a complex of this invention formed of the MT-β subunit with cisplatin (beta-cis) as discussed in Example 8 below.

FIG. 9 is a bar graph indicating the results of a Caspase-3 induction assay of MT and complexes of MT subunits and cisplatin in into DMS53 small cell lung cancer cells as discussed in Example 8 below. The symbols are as stated in FIG. 8.

FIG. 10 is a bar graph illustrating the results of a 4 hour uptake into J82 transitional cell bladder cancer cells of phosphate buffered saline (PBS), cisplatin alone (cis), metallothionein alone (MT), a complex formed of MT and cisplatin (MT-cis), a complex of this invention formed of the MT-α subunit only with cisplatin (alpha-cis) and a complex of this invention formed of the MT-β subunit with cisplatin (beta-cis) as discussed in Example 8 below.

FIG. 11 is a bar graph indicating the results of a Caspase-3 induction assay of MT and complexes of MT subunits and cisplatin in J82 transitional cell bladder cancer cells as discussed in Example 8 below. The symbols are as stated in FIG. 10.

FIG. 12 is a bar graph indicating ovarian cancer OVCAR3 cell uptake of metallothionein. OVCAR3 cells grown to confluence in 96 well plates were exposed to 40μM Cy3-fluorescently conjugated MT for 1,2 and 4 hours. Cells were washed, surface binding released with acid, and trypsinized for flow cytometry analysis of MT uptake.

FIG. 13 is a bar graph showing that cisplatin and MT-complexed cisplatin kill OVCAR3 cells. OVCAR3 cells grown to confluence in 96 well plates were exposed to varied concentrations of cisplatin or MT-complexed with cisplatin and cell death assayed by trypsinizing the cells off the beads, filtering off the beads, and staining with propidium iodide for membrane integrity. Propidium iodide uptake was assayed by flow cytometry.

FIG. 14 is a graph showing time course of caspase-3 activity after cisplatin treatment of J82 transitional carcinoma cells, using cisplatin alone or cisplatin conjugated to the β-subunit of MT. Values shown are mean ± standard error, n=6. Data files of 2,000 observations/sample were collected by flow cytometry.

DETAILED DESCRIPTION OF THE INVENTION

In response to the need of the art for additional therapeutic compositions and methods for the treatment of cancer with heavy metal therapeutics and for the treatment of heavy metal poisoning, the inventors identified the receptor in the proximal kidney tubules to which naturally occurring MT (optionally laden with heavy divalent metal ions) binds. The binding between the MT complexed with a heavy metal ion and megalin causes the MT-heavy metal complex to remain in high concentration in the renal tubules, resulting in nephrotoxicity and renal tubule damage. Using this discovery, the inventors then provided the following compositions for meeting the above-identified needs of the art.

A. The Metallothionein Receptor in the Renal Tubules

The inventors have determined that megalin binds metallothionein and is the most important mechanism of MT uptake in to the renal proximal tubule. The inventors have further identified the highly conserved hinge or interdomain region of metallothionein, centered on a lysine repeat, as the critical site for binding to megalin.

(1) Identification of the Receptor

Megalin is the most abundant protein in the proximal tubule of the kidney. It is heavily expressed in S_1 in the peri-glomerular area, and is a multi-ligand receptor with multiple binding sites. Megalin has 4 binding sites, and cubilin at least

27 domains and 8 EGF repeats as binding sites, and yet these two proteins are thought to be largely responsible for the reabsorption of an immense volume of diverse ligands in the proximal tubule (Saito A et al, 1994 *Proc Natl Acad Sci USA* 91: 9725–9729; Verroust PJ. et al, 2002 Kidney Int 62: 745–756, Verroust PJ and Kozyraki R. 2001 *Curr Opin Nephrol Hypertens* 10: 33–38). Given the long list of ligands for megalin, and the abundance of these proteins in the glomerular filtrate, the effectiveness of the uptake likely relies on the very large content of megalin in the kidney (Verroust PJ and Kozyraki R. 2001 *Curr Opin Nephrol Hypertens* 10: 33–38). On simple SDS-PAGE gels of renal proximal tubular brush borders, the two most abundant proteins are the distinctive 460and 600-kDa molecular masses of cubilin and megalin (Verroust PJ. et al, 2002 Kidney Int 62: 745–756). When one combines the abundant expression of megalin with the large surface area created by brush-border formation, there is abundant megalin to facilitate reabsorption of all available ligands (Verroust PJ. et al, 2002 Kidney Int 62: 745–756, Verroust PJ and Kozyraki R. 2001 *Curr Opin Nephrol Hypertens* 10: 33–38).

Experiments described in the examples below support the determination that binding of MT to megalin is critical in renal proximal tubular uptake of MT-bound heavy metals. First, MT binds megalin, but not cubilin, in direct surface plasmon resonance (SPR) studies in a dose, ion and pH dependent manner. Binding of MT occurs at a single site with a Kd ~10^{-4} and, as with other megalin ligands, depends on divalent cations. Second, antisera and various known megalin ligands inhibit the uptake of fluorescently labeled MT in model cell systems. Anti-megalin antisera, but not control sera or anti-NK-1 receptor, displace >90% bound MT from rat renal brush-border membranes. Megalin ligands, including β2-microglobulin and also recombinant MT fragments, identified below, compete for uptake by megalin-expressing rat yolk sac BN-16 cells. Third, megalin and fluorescently labeled MT colocalize in BN-16 cells, as shown by fluorescent microscopic techniques.

The distribution and molecular characteristics of megalin are consistent with these results of metallothionein uptake. Taken together with the observation that megalin mediates uptake of β2-microglobulin, these result provides evidence

implicating megalin in metallothionein uptake. The results illustrated in the examples and data herein are also consistent with the binding properties and physiological observations of megalin and its ligands in other systems. The dissociation constant estimated at 9.8 x 10^{-5} M may appear small for a receptor-ligand interaction, but it is similar to values obtained for other known megalin ligands (Gburek J, et al, 2002 *J Am Soc Nephrol* 13: 423–430). The calcium dependence of MT binding is also consistent with similar ion requirements of other megalin ligands (Birn H, et al, 1997 *J Biol Chem* 272: 26497–26504; Moestrup SK. et al, 1998 *J Biol Chem* 273: 5235–5242), but the dependence on magnesium is unusual. The inventors' observation of competition between β2-microglobulin and MT binding to megalin establishes that MT and β2-microglobulin compete directly for renal uptake in live animals through competition for megalin binding. Diverse MTs bind megalin with the same kinetics and may be important to ensure efficient reuptake of diverse isoforms in the proximal tubule.

(2) Identification of the Receptor-MT Critical Binding Site

The initial analysis of the MT/megalin binding site used a peptide library, because the midregion of the MT molecule models to a linear peptide with little secondary or tertiary structure. MT is so highly conserved across species and phyla that comparison of protein isoforms from different species (even the naturally occurring isoforms of MT from Ia and Ib, through II, III, IV, V, and others) was not useful in defining binding sites (Atrian S, et al., "Recombinant synthesis and metal-binding abilities of mouse metallothionein 1 and its α-and β-domains." In: *Metallothionein IV*, ed. Klaassen CD. Basel: Birkha¨user Verlag, 1999, p. 55–61; Huang PC et al, "Native and engineered metallothioneins." In: *Metallothionein in Biology and Medicine*, ed. Klaassen CD and Suzuki KT., Boca Raton, FL: CRC, 1991, p. 87–101; and Kägi JHR and Vasak M., "Chemistry of mammalian metallothionein." In: *Metallothionein in Biology and Medicine*, eds. Klaassen CD and Suzuki KT., Boca Raton, FL: CRC, 1991, p. 49–60.)

Using follow-up SPR and flow cytometry studies with overlapping MT peptides and recombinant MT fragments, the inventors identified the hinge region SCKKSCC (aa 28-34 of SEQ ID NO: 1) of MT as one critical site for megalin

binding. In lower species, MT exists as two separate molecules, binding three and four heavy metal moieties. However, in mammals and other higher organisms the two molecules have coalesced, joined by a hinge region centered on a highly positively charged lysine repeat. The hinge region sequence SCKKSCC (aa 28-34 of SEQ ID NO: 1) is even more heavily conserved than the rest of the MT sequence, being identical in virtually all known mammalian species, and all the various MT isoforms in each species (Huang et al, cited above; Kägi and Vasak, cited above). Studies using site-directed mutagenesis have established the critical role of the conserved lysine repeat of the hinge region in the detoxification function of MT in yeast. Replacement of one or both lysines in the hinge or interdomain region was inconsequential to the structure and function of MT unless both substituted residues are uncharged (Cody CW and Huang PC. 1993 *Biochemistry* 32: 5127–5131; Cody CW and Huang PC. 1994 *Biochem Biophys Res Commun* 202: 954–959). Numerous investigators reported extremely low yields and splice variants when attempting to express MT (Atrian, cited above), thereby implying that site-directed mutagenesis and expression of mutated MT isoforms was not likely to be useful.

After the failure of non-MT-derived peptides with a central KK motif to inhibit megalin-MT interaction in direct SPR studies of the examples implicated the interdomain of MT as a binding site, the inventors investigated recombinant MT subunits (Atrian et al), which disrupt the candidate binding motif. Recombinant production of MT fragments dividing MT at the lysine-lysine hinge yields intact α- and β-subunits that still bind heavy metals (Atrian, cited above). The inventors' observations of charged peptides and the highly charged polybasic antibiotic gentamicin suggest more structural requirements than simply charge for a molecule to interfere with the megalin-MT interaction.

The following compositions and methods of this invention are based on the discovery that disruption of the SCKKSCC motif inhibits proximal tubular MT uptake and thereby eliminates much of the renal accumulation and toxicity of heavy metals, such as cadmium, gold, copper, and cisplatinum.

B. Compositions of the Invention

In view of the above-noted discovery of the MT receptor and binding site *in vivo*, the inventors have determined that mutants of metallothionein that bind heavy metals, but do not bind megalin, or that bind megalin with reduced avidity compared to MT, are not taken up by proximal tubular cells. Thus, such mutant MTare useful in the field of heavy metal therapeutics. More specifically, the inventors provide herein compositions that can deliver a therapeutic heavy metal, e.g., preferably anti-neoplastic platinum compounds, on a mutated MT which does not bind megalin. This composition and uses thereof avoid therapy-limiting nephrotoxicity of heavy metals used as anticancer agents.

A composition of the present invention is a conjugate formed by (1) a modified metallothionein (MT) amino acid sequence or fragment thereof that binds the megalin receptor less avidly than naturally-occurring MT and that binds a divalent metal ion or heavy metal molecule and (2) multiple molecules of a therapeutic divalent metal ion.

With regard to the modified MT sequence of this conjugate, the term "binding less avidly than naturally-occurring MT" as used herein is defined as a binding affinity between the modified MT and megalin of less than Kd $\sim 10^{-5}$ to a Kd of 0. Such a binding affinity is low enough that the naturally-occurring MT *in vivo* would successfully compete with the modified MT for binding to megalin. Such a low binding affinity also includes no binding of the modified MT amino acid sequence of this invention to megalin receptor at all. More specifically, any modification to MT sequences that involves disruption of the SCKKSCC hinge region sequence of naturally-occurring MT will provide a sequence that meets the requirements of this invention.

(1) Modified Full-Length MT Sequences of the Invention

Thus, an exemplary modified MT sequence is either a full-length MT sequence or modified full-length sequence of naturally-occurring MT in which the hinge region is disrupted. For example, one embodiment of a modified MT of this invention includes a sequence of formula

$MDPNC_1SC_2ATGNSC_3TC_4ASSC_5KC_6KEC_7KC_8TSC_9X\ X'SC_{10}C_{11}SC_{12}C_{13}PAGC_{14}$ $TKC_{15}AQGC_{16}IC_{17}KGASDKC_{18}SC_{19}C_{20}A$, SEQ ID NO: 4, wherein X and X' are independently selected from any amino acid other than K. In another embodiment includes the sequence of SEQ ID NO: 4, in which X and X' are any uncharged amino acids. In another embodiment includes the sequence of SEQ ID NO: 4, in which X and X' are any negatively charged amino acids. Still another embodiment of a modified MT of this invention includes SEQ ID NO: 4 in which in which X and X' are non-naturally occurring amino acids, as defined below. Still another embodiment of a modified MT of this invention includes SEQ ID NO: 1 in which in which X-X' include a spacer or bridge of one or more naturally-occurring or non-naturally-occurring amino acids, carbohydrate moieties or other chemical moieties interposed between the amino acid residues of X and X', wherein X and X' may be any amino acid, including lysine. The spacer or bridge is of any size or composition sufficient to disrupt the positive charge formed by the KK sequence.

Preferably, any modification of the KK sequences that disrupts the strong positive charge at that position in the MT sequence will provide a modification sufficient to prevent binding of the modified MT to the megalin receptor. For example, modifications that place a negative charge on the MT sequence at that point are useful. Modifications of the full-length MT sequence that convert the positive KK cloud at the hinge region into a neutral 'charge' are also useful.

Additionally, any modification of the MT sequence that enlarges the complex formed by the MT bound to the heavy metal molecules to a size that cannot fit through the renal glomeruli is useful for this purpose. Preferably the size of the modified MT in this embodiment is larger than the ~70kD sieves of the kidney. Thus, included in the modification of full-length MT includes conjugates of the modified full-length MT, such as multiple modified full-length MT fused together to form a larger protein sequence. Also included as modifications full-length MT are large proteins formed by modified full-length MT which are modified by chemical or carbohydrate moieties to form a large protein that cannot fit through the renal glomeruli. Molecules that do not fit through the renal glomeruli will be filtered through the liver rather than the kidney. Such compounds are not toxic to the liver.

A further modified MT sequence includes SEQ ID NO: 4 in which X and X' are selected from any of the embodiments above and in which all cysteine residues are invariant, and further in which any of the non-C residues are substituted by non-naturally occurring amino acids, as defined below. Such non-naturally occurring amino acids can contribute to changing the charge or size of the modified full-length MT, to fit the parameters described above.

In other embodiments of a modified full-length MT sequence of this invention, and in any of the embodiments described above, X and X' are both glutamine residues.

(2) Modified Fragments of MT Sequences of the Invention

Another exemplary modified MT sequence is a fragment of the naturally-occurring MT sequence in which the hinge region is disrupted. As one example, a modified MT sequence comprises a modified β-MT subunit sequence of the formula $MDPNC_1SC_2ATGNSC_3TC_4ASSC_5KC_6KEC_7KC_8TSC_9X$ SEQ ID NO: 2, wherein X is any amino acid other than K. In another embodiment, the MT sequence is SEQ ID NO: 2, wherein X is an uncharged amino acid other than K. In still another embodiment, X can be a negatively-charged amino acid. In still another embodiment, the MT sequence is SEQ ID NO: 2, wherein X is a non-naturally occurring amino acid, as defined below. In yet a further embodiment, the modified MT sequence is SEQ ID NO: 2 in which X is selected from any of the embodiments above and in which all cysteine residues are invariant, and further in which any of the non-C residues are substituted by non-naturally occurring amino acids, as defined below.

Yet a further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 2, in which X is defined as any of the above-noted suggestions and which is truncated at the amino terminus, leaving as the first amino terminal amino acid, the residue C_1. A further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 2, in which X is defined as any of the above-noted suggestions and which is truncated at the amino terminus, leaving as the first amino terminal amino acid, the residue C_2. A further embodiment

of a modified MT fragment according to this invention includes SEQ ID NO: 2, in which X is defined as any of the above-noted suggestions and which is truncated at the amino terminus, leaving as the first amino terminal amino acid, the residue C_3. A further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 2, in which X is defined as any of the above-noted suggestions and which is truncated at the amino terminus, leaving as the first amino terminal amino acid, the residue C_4.

Other fragments of this invention may include the β subunit of MT, truncated at the carboxy terminus. Truncations of the subunit must be sufficient to permit at least one divalent heavy metal to be carried by the modified MT fragment.

Another exemplary modified fragment of the naturally-occurring MT sequence in which the hinge region is disrupted includes a modified α - MT subunit sequence of the formula $X'SC_{10}C_{11}SC_{12}C_{13}PAGC_{14}TKC_{15}AQGC_{16}IC_{17}KGASDKC_{18}SC_{19}C_{20}A$, SEQ ID NO: 3, wherein X' is any amino acid other than K. In another embodiment, the MT sequence is SEQ ID NO: 3, wherein X' is an uncharged amino acid other than K. In another embodiment, the MT sequence is SEQ ID NO: 3, wherein X' is a negatively charged amino acid. In still another embodiment, the MT sequence is SEQ ID NO: 3, wherein X' is a non-naturally occurring amino acid, as defined below. In yet a further embodiment, the modified MT sequence is SEQ ID NO: 3 in which X' is selected from any of the embodiments above and in which all cysteine residues are invariant, and further in which any of the non-C residues are substituted by non-naturally occurring amino acids, as defined below.

Yet a further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 3, in which X' is defined as any of the above-noted suggestions and which is truncated at the carboxy terminus, leaving as the last carboxy terminal amino acid, the residue C_{20}. A further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 3, in which X' is defined as any of the above-noted suggestions and which is truncated at the carboxy terminus, leaving as the last carboxy terminal amino acid, the residue C_{19}. A further embodiment of a modified MT fragment according to this invention includes SEQ ID

NO: 3, in which X' is defined as any of the above-noted suggestions and which is truncated at the carboxy terminus, leaving as the last carboxy terminal amino acid, the residue C_{18}. A further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 3, in which X' is defined as any of the above-noted suggestions and which is truncated at the carboxy terminus, leaving as the last carboxy terminal amino acid, the residue C_{17}. A further embodiment of a modified MT fragment according to this invention includes SEQ ID NO: 3, in which X' is defined as any of the above-noted suggestions and which is truncated at the carboxy terminus, leaving as the last carboxy terminal amino acid, the residue C_{16}. Modified α-MTs of this invention may also include the sequences truncated at the amino terminus. Truncations of the subunit must be sufficient to permit at least one divalent heavy metal to be carried by the modified MT fragment.

Still other embodiments of a modified αMT or βMT fragment according to this invention includes any of the above embodiments, in which any of the non-C residues are substituted by non-naturally occurring amino acids, as defined below.

In other embodiments of a modified α or β MT fragment of this invention, and in any of the embodiments described above, X or X' is a glutamine residue.
Still other embodiment of a modified MT fragment according to this invention includes any of the above embodiments of the modified α- or β-MT subunits, in which any of the non-C residues are substituted by non-naturally occurring amino acids, as defined below. Still other fragments of these modified subunits are those in which one or more amino acids are attached to chemical or carbohydrate moieties sufficient to increase the size of the fragment to larger than will be filterable by the kidneys, or that will retain a neutral or negative charge. As described above, among such additional modifications are conjugates of multiple copies of the β-subunit of MT, multiple copies of the α-subunit of MT, or conjugates of one or more copies of the β-subunit of MT with one or more copies of the full-length modified MT, or conjugates of one or more copies of the modified β-MT with one or more copies of the α-MT, or conjugates formed by one or more copies of the modified β-MT with one or more copies of the modified α-MT and one or more copies of the modified full-length MT. Such conjugates may be formed of each modified MT sequence or fragment fused

directly to a terminal amino acid of another modified MT sequence or fragment. Such conjugates may be formed of each modified MT sequence or fragment fused indirectly to another modified MT sequence or fragment via a chemical, amino acid or carbohydrate linker. Desirably, such conjugates are greater in size than the size of the kidney sieves, e.g., greater than 70kD in size when coupled with the appropriate heavy metals.

(3) Naturally and Non-Naturally Occurring Amino Acids

"Naturally-occurring amino acid" is used herein to refer to the twenty amino acids that occur in nature in L form, which include alanine, cysteine, aspartate, glutamate, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, aspargine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine, or any derivative thereof produced through a naturally-occurring biological process or pathway.

"Non-naturally-occurring amino acid" is used herein to refer to an amino acid other than a naturally-occurring amino acid as defined above, which can be synthesized or "man-made", and including a derivative thereof, whether produced synthetically or via a biological process or pathway. Non-naturally occurring amino acids include, without limitation, D amino acids, amino acids containing unnaturally substituted side chains, e.g., methyl-Arg, cyclic amino acids, diamino acids, ß-amino acids, homo amino acids. Non-naturally-occurring or unnatural amino acids may be characterized by novel backbone and side chain structures and are widely available from commercial reagent suppliers, such as Sigma-Aldrich (www.sigmaaldrich.com), www.Netchem.com and other sites. See also a broad literature on such structures including, without limitation, Han S and Viola RE, Protein Pept. Lett. **2004** 11(2):104-14; Ishida et al, Biopolymers **2004** 76(1):69-82; Sasaki et al, Biol. Pharm. Bull. **2004** 27(2):244-7; Pascal R et al, Meth. Enzymol. **2003** 369:182-94; Yoder NC and Kumar K, Chem. Soc. Rev. **2002** 31(6):335-41; and Ager DJ, Curr. Opin. Drug Discov. Devel. **2002** 5(6):892-905, among others, which are incorporated herein by reference. This term does not encompass those derivatives which fall within the definition of a "naturally-occurring amino acid", as defined above.

Such non-naturally occurring amino acid(s) when employed in the compounds above are anticipated to make the compounds more resistant to degradation by mammalian enzymes in serum, saliva, stomach and intestines, and thus compounds that are composed of one or more such amino acids may confer upon the compound enhanced stability and bioavailability *in vivo*. A variety of methods for producing non-natural amino acids are known and may be selected by one of skill in the art. For example, one class of non-naturally occurring amino acids are L amino acids that effect stereochemistry. Thus, in one embodiment of compounds of this invention, one or more of the amino acids in the peptide may be in L form, while others may be in D form. Another non-naturally occurring amino acid is an amino acid which is modified to contain a substitution on the alpha-carbon in the amino acid structure. For example the alpha-carbon may be substituted by a suitable hydrocarbon moiety, such as aminoisobutyrate. Still another class of non-naturally occurring amino acids is amino acids which are modified or mutated to extend their carbon chain length. For example, an amino acid with a single alpha-carbon chain, may be extended with at least one additional carbon, i.e., a beta-carbon, and so on. An additional modification to an amino acid is the insertion of a substituent on the nitrogen of the amino group. An example of this type of modification is an N-methyl amino acid. The addition of substituents on the alpha carbon or additional carbons or on the nitrogen of the amino acid molecule may occur in any of the amino acids of the formula above.

Among useful substituents for creating the non-naturally occurring amino acids are a straight chain, branched, cyclic or heterocyclic C_{1-12} alkyl group, and straight chain, branched, cyclic, or heterocyclic C_{1-12} alkanoyl group. The amino acid may be also modified by the insertion of modifying sugars, imide groups and the like. Other amino acids are substituted in the ortho or meta position by a substituent such as H, OH, CH_3, halogen, OCH_3, NH_2, CH or NO_2.

A non-exclusive list of modified or non-naturally occurring amino acids for inclusion in compounds fitting the formula above include amino acids modified by N-terminal acetylation, C-terminal amidation, formylation of the N-terminal methionine, gamma-carboxyglutamic acid hydroxylation of Asp, Asn, Pro or Lys residues in the compound, methylation of Lys or Arg, preferably; phosphorylation of Ser, Thr, Tyr,

Asp or His in the compound, use of a pyrrolidone carboxylic acid, which is an N-terminal glutamate which has formed an internal cyclic lactam, sulfatation of Tyr, generally. Still other modifications of non-naturally occurring amino acids include use of or substitution with the following moieties: a 2-aminoadipic acid group, a 3-aminoadipic acid group, beta-Ala or beta-aminopropionic acid group, 2-aminobutryic acid, 4-aminobutyric acid, piperidinic acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutryic acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2, 4 diaminobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylglycine, N-ethyl asparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, 6-N-methyllysine, N-methylvaline, 6-N-methyllysine, norvaline, norleucine, and ornithine.

One of skill in the art may readily select among such non-naturally-occurring amino acids to modify the MT sequences or fragments to provide the modified MT sequence or fragment with the appropriate or no binding affinity to megalin according to this invention.

A variety of publications directed to MT derivatives used in binding studies propose modified MT sequences and fragments that may also be useful in the methods and compositions of this invention. Such publications include, among others, Tio et al, 2004, J. Biol. Chem., 279(23):24403-24413; Pan et al, 1999 Eur. J. Biochem., 266:33-39; Romero-Isart et al, 1999 Eur. J. Biochem., 259:519-527; Nielson and Winge, 1984 J. Biol. Chem., 259 (8):4941-4946; Nielson and Winge, 1985 J. Biol. Chem., 260(15):8698-8701; Valls et al, 2001 J. Biol. Chem., 276(35):32835-32843, all incorporated herein by reference.

(5) Divalent Metal Ions or Heavy Metals

As a further component of compositions of this invention, the modified MT sequence defined above are conjugated or associated with at least one or multiple molecules of heavy metals or divalent metal ions. Thus, compositions of this invention desirably contain in association with the modified MT sequence from one to seven molecules of a divalent metal ion. In one embodiment, an MT fragment is conjugated to three divalent metal ions. In another embodiment the MT fragment is

conjugated to four divalent metal ions. Preferably a β-MT fragment is associated with three metal ions. Preferably a α-MT fragment is associated with four metal ions. Where the modified MT sequences are conjugated together, the number of molecules of divalent metal ion are multiples of the one to seven molecules carried by each individual modified MT sequence.

Desirable divalent metal ions for such use include, without limitation, anti-neoplastic platinum compounds, gold, copper, and cadmium. Known anti-neoplastic platinum compounds include cisplatin, oxyplatin and carboplatin. See, e.g., other compounds disclosed in Platinum-Based Drugs in Cancer Therapy, eds. Kelland and Farrell (March 2000) ISBN No. 1-59259-012-8, incorporated herein by reference. Still other metal compounds that bind to the cysteine binding sites on metallothionein and are useful in therapeutic treatments of disease, e.g., cancers, are also anticipated to be useful in the compositions and methods of this invention. In one specific embodiment exemplified in Examples 7-8 below, a composition of this invention includes a β-MT fragment in which X is Q and which is associated with three molecules of cisplatin. One of skill in the art, given this disclosure, may readily prepare other embodiments of this invention.

6. Optional Delivery Peptides

In other embodiments of this invention, the modified MT sequences conjugated with divalent metal ions may be further associated with other peptides or proteins for the purpose of focusing the delivery of the conjugate to a desired target site. Therefore, in one embodiment, the composition includes a delivery peptide for targeted delivery to a desired cell, preferably a tumor cell. The delivery peptide is preferably fused to the modified MT or fragment thereof at one or the amino or carboxy termini. Preferably the delivery peptide is attached at a point in the MT sequence that least disrupts the binding of the heavy metal molecules.

For example, such a delivery peptide may be a penetration enhancer or transport sequence, a "cell penetrating peptide" (CPP) or "protein transduction domain" (PTD). Some examples of suitable CPPs are arginine-rich peptides, and more specifically, linear or branched-chain peptides containing approximately 8 residues of arginine (See, e.g., Futaki et al Curr. Prot. Pept. Sci., 2003 4(2):87-96;

and Futaki Int. J. Pharm, 2002 245(1-2):1-7, both incorporated by reference herein). Other suitable CPPs are also discussed in International Published Patent Application Nos. WO 03/035892 and WO 03/035697.

Other delivery peptides for fusion to the compositions of this invention include transactivating protein analogs or fragments thereof, such as the HIV-1 Tat (Vives et al, Curr. Protein Pept. Sci., 2003 4(2):125-32). The HIV-1 Tat basic peptide sequence is an example of the prototypic cell membrane-permeant component. US Patent No. 6,348,185 refers to cell membrane-permeant peptides including peptides of 4 to 6 amino acids derived from HIV-1 Tat, linked to pharmaceutically active substances via a functional linker that confers target cell specificity to the composition. U.S. Patent Nos. 5,804,604; 5,747,641; 5,674,980; 5,670,617; 5,652,122 (Frankel) refers to the use of Tat peptides to transport covalently linked biologically active cargo molecules into the cytoplasm and nuclei of cells. Morris *et al, Nat. Biotechnol.*, 2001 *19*(12):1173-76 refers to PTDs including TAT protein sequences. US Patent No. 5,804,604 refers to Tat-derived transport polypeptides. A commercial useful peptide transport molecule is the CHARIOT™ reagent (Active Motif).

Still other options for the transport peptides useful in the present invention are described in U.S. Patent Nos. 5,135,736 and 5,169,933 (Anderson), which refer to the use of covalently linked complexes (CLCs) to introduce molecules into cells. Yet another embodiment of a delivery peptide is a peptide-oligodeoxynucleotide conjugate described by L. Chaloin *et al, Biochem.*, 1997 *37*:11179-87. These conjugates comprise the combination of a peptide containing a hydrophobic motif associated with a hydrophilic nuclear localization sequence covalently linked to a small molecule to facilitate the cellular internalization of small molecules. The hydrophobic sequences used correspond to a signal peptide sequence or a fragment of the fusion peptide GP41. One peptide successfully targeted fluorescent oligodeoxynucleotides into living cells (Chaloin *et al*, Biochem. Biophys. Res. Commun., 1998 243(2):601-608). Still another suitable transport peptide is described by Taylor *et al, Electrophoresis*, 2003 *24*(9):1331-1337 and refers to an amphipathic

peptide Pep-1 which may be used as a transport peptide in combination with a nonionic detergent carrier, for delivery of SDS-PAGE isolated proteins into a cell.

The delivery or transport peptide useful in the present invention can be any cell membrane-permeant basic peptide component of the complexes described in the above-cited documents, all of which are incorporated by reference herein. The transport peptide can be a peptide or protein that comprises any amino acid sequence (including naturally-occurring amino acids or non-natural amino acids, such as D amino acids) that confers the desired intracellular translocation and targeting properties to the selected therapeutic peptide or protein. Preferably, these amino acid sequences are characterized by their ability to confer transmembrane translocation and internalization of a complex construct when administered to the external surface of an intact cell. Attachment of a compound of the formula of the present invention to the delivery peptide would permit the resulting composition to be localized within cytoplasmic and/or nuclear compartments.

Specific delivery peptide sequences useful in practicing the present invention include, but are not limited to, sequences of the following proteins and fragments and homologous sequences derived therefrom: the HIV-1 Tat protein, the HIV-1 Rev protein basic motif, the HTLV-1 Rex protein basic motif, the third helix of the homeodomain of Antennapedia, a peptide derivable from the heavy chain variable region of an anti-DNA monoclonal antibody, the Herpes simplex virus VP22 protein, the Chariot™ protein, and the Pep-1 protein. The minimum number of amino acid residues can be in the range of from about three to about six, preferably from about three to about five, and most preferably about four.

The delivery peptide sequence attached to the modified MT-metal complex can also contain an enzymatic cleavage site for interposition between the delivery peptide sequence and the MT sequence of the complexes described above. This optional sequence permits the delivery peptide to be cleaved from the MT sequence intracellularly, if desired. Selection of such cleavage sites is within the skill of the art.

Any of the compositions specifically identified herein and others within the teachings of this specification can all be readily tested for the required biological function, e.g., the ability to bind megalin less avidly than naturally occurring MT or

not at all and the ability to complex with multiple molecules of divalent metal ions, as well as for the ability to deliver the metal ion to a target in mammalian cells and tissues *in vitro* and *in vivo*. The resulting composition may be screened for biological activity and/or metabolic stability by *in vitro* and in *vivo* assays, such as those described in the examples and in the art. These compounds generally have "significant" metabolic stability in mammalian serum, i.e., the compounds are stable for at least 2 hours in serum. More preferred compounds are stable for at least 4 hours in serum. Still more preferred compounds of this invention are stable in serum for greater than 8 hours.

C. Methods of Manufacture

Compounds and conjugates of the invention may be prepared conventionally by known chemical synthesis techniques. Compounds of the invention may also be purchased from a commercial vendor, e.g. the Sigma-Aldrich Co. Among such preferred techniques known to one of skill in the art are included the synthetic methods described by Merrifield, J. Amer. Chem. Soc., 1963 85:2149-2154; and in texts such as G. C. Barrett and D. T. Elmore, "Amino Acids and Peptides" Oct. 1998; and "Peptides: Chemistry and Biology", eds. N. Sewald, H-D Jakubke, Aug. 2002; and other conventional textbooks relating to the construction of synthetic compounds.

Such compositions may be produced recombinantly by conventional methods. Specific embodiments of compounds of this invention are disclosed in detail in Example 7 below.

Such methods of producing and assembling the individual components of this invention, as well as the conjugates and pharmaceutical compositions described herein may use the techniques described in the examples or other techniques in the art. For instance, as demonstrated by Example 7, a method of producing a composition as described above, a modified MT amino acid sequence or fragment thereof that does not bind megalin is prepared by recombinant methods or purchased. Thereafter, the MT sequence was combined in a suitable ratio in a suitable buffer and incubated for a time sufficient to replace any divalent metal ions on the MT with the

desired metal ion. In an alternate step, the selected metal ion may be complexed with the modified MT by raising the pH to complex the selected metal ion to the sequence.

The ratio of metallothionein in its native embodiment to heavy metals is 1:7. In one embodiment, the ratio of metallothionein to a heavy metal, e.g., cisplatin by weight is about 5:3.. Any suitable ratio may be applied depending upon the modified MT selected, the heavy metal ion selected, and the number of binding sites for the heavy metal available on the modified MT. One of skill in the art may readily select a suitable ratio.

Incubation temperature can range from about 25 to about 42°C . In one embodiment, the temperature is about 37°C. Still other ranges may be readily selected by one of skill in the art. Similarly, the incubation time can range from 60 minutes to 48 hours. In one embodiment, the incubation time is 48 hours. Still other ranges may be readily selected by one of skill in the art.

Any number of conventional buffers may be selected for this use depending upon the functional requirements of the formulation as determined by one skilled in the art. See, e.g., buffers listed in Good, N. E. et al. (1966) *Biochemistry 5*, 467 and Good, N. E., and Izawa, S. (1972) *Methods Enzymol. 24*, 53 The buffer may be selected from a variety of buffers known to those of skill in the art to be used in the compositions of the invention and include, without limitation, phosphate buffered saline (PBS) or isotonic saline, such as ISOTON II (US Patent 3,962,125), Tris buffer, the organic buffer N-(2-Acetamido)-2-iminodiacetic acid (ADA), or pyrophosphate buffer, acetate buffers, succinate buffers, maleate buffers, citrate buffers, imidazole buffers, carbonate buffers, MES buffer, MOPS buffer, and HEPES buffer, among many that may be readily selected by one of skill in the art. In one embodiment, the buffer was 10mM HEPES buffer. Thereafter the resulting product is dialysed against another suitable buffer, e.g., PBS or carbonate buffer.

Alternative steps for preparation can include stripping all heavy metals from the MT sequence by placing the sequence into solution in a suitable buffer, e.g., 10mM HEPES buffered to pH 3.0, and degassing the solution with dry nitrogen to strip all heavy metals. A desired heavy metal, e.g., zinc, may be conjugated to the

MT sequence by dialysis against a buffer. One such buffer contains 2mM beta-mercaptoethanol, and 1mM zinc chloride in 10mM HEPES pH 7.4

One of skill in the art may readily assemble the compositions and components of this invention given the teachings provided herein, without resorting to undue experimentation.

D. Pharmaceutical Formulations

Pharmaceutical compositions of the present invention, in one embodiment, contain a modified MT sequence conjugated to molecules of a divalent metal ion or components thereof as described above in a pharmaceutically acceptable carrier with other optional suitable pharmaceutically inert or inactive ingredients. In another embodiment, pharmaceutical compositions of the present invention contain one or more compositions, components or conjugates described above or with one or more different therapeutically useful reagents. In one embodiment, a single modified MT-divalent metal ion complex is present in a single composition. In another embodiment, two or more different modified MT-divalent metal ion complexes are combined with one or more chemotherapeutic agents and/or biological agents, radiological agents, and/or other therapeutic agents as described below.

The pharmaceutical compositions of this invention include the modified MT-metal ion complexes of this invention formulated neat or with one or more pharmaceutical carriers for administration, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmacological practice. The pharmaceutical carrier(s) may be solid or liquid. Formulations may incorporate both solid and liquid carriers.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. Suitable solid carriers

include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins, crystalline cellulose, binders such as hydroxypropylmethyl cellulose, coating agents such as hydroxypropylmethyl cellulose and terephthalate thereof, lubricants such as zinc stearate and aluminum stearate.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixers and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. Carriers include glycerol, propylene glycol, liquid polyethylene glycol, and the like. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, isotonic agents, sugars, sodium chloride, anti-oxidants, buffers, bacteriostats, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, surfactants, colors, viscosity regulators, stabilizers or osmo-regulators, tensio-active agents. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, lethicins, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant, solubilizing and dispersing agents such as polyoxyethylene hardened castor oil, stabilizers, pH adjusters, and isotonicity-imparting agents, preservatives, anti-bacterial and anti-fungal agents, liposomes, mannose, glucose and balanced salt solutions, phosphate buffered saline, diethyl ether, isopropyl ether, halothane, or trifluorotrichloroethane.

Additionally, the compositions of the present invention may be formulated alone in a composition of the invention or may be formulated in combination with

other compounds of the invention and other compounds known in the art of cancer treatment. Compounds of the present invention may also be used in combination with other therapeutic agents. In certain embodiments, such other such agents include, but are not limited to chemotherapeutic agents, such as anti-metabolites, DNA damaging, microtubule destabilizing, microtubule stabilizing, actin depolymerizing, growth inhibiting, topoisomerase inhibiting, HMG-CoA inhibiting, purine inhibiting, pyrimidine inhibiting, metaloproteinase inhibiting, CDK inhibiting, caspase inhibiting, proteaosome inhibiting, angiogenesis inhibiting, differentiation inducing and immunotherapeutic drugs, and compositions for promotion of TGF-β response and/or apoptosis. These agents include, but are not limited to, anthracycline antibiotics such as doxorubicin and mitoxantrone, estramustine, vinblastine, paclitaxel, etoposide, cyclophosphamide, cisplatin, carboplatin, adriamycin, 5-fluorouracil, camptothecin, actinomycin-D, mitomycin C, adriamycin, verapamil, podophyllotoxin, and the like (with or without the addition of steroid drugs); anti-androgens (such as flutamide, bicalutamide, nilutamide, megestrol acetate, adrenocorticotropic hormone secretion inhibitors, ketoconazole, estrogens, anti-estrogens and LHRH production suppressors), immunomodulatory agents (including cytokines, chemokines, interferons, interleukins), a non-progestin/non-estrogen apoptosis promoting agent selected from the group consisting of the retinoids (retinoic acid, N-(4-hydroxyphenyl) retinamide-O-glucuronide, N-(4-hydroxyphenyl) retinamide, O-glucuronide conjugates of retinoids, N-(4-hydroxyphenyl) retinamide and its glucuronide derivative, retinyl-β-glucuronide, the glucuronide conjugates of retinoic acid and retinol, tretinoin, etretinate, arotinoid, isotretinoin, retinyl acetate, acitretin, adapalene, and tazarotene), adamantyl or adamantyl group derivatives containing retinoid-related compounds (e.g., 6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid, 2-[3-(1-adamantyl)-4-methoxyphenyl]-5-benzimidazole carboxylic acid, and 6-[3-(1-adamantyl)-4,5-methylenedioxyphenyl]-2-naphthoic acid; 2-methoxyestradiol), progestin, 1-O-acetylbritannilactone, 1,6-O,O-diacetylbritannilactone, a 2-nitroimidazole derivative (e.g. 1-(2,3-dihydroxy-1-(hydroxymethyl)-propoxymethyl)-2-nitroimidazole, 1-(4-hydroxy-2-butenyloxymethyl)-2-nitroimidazole and 1-(2,3-dihydroxypropoxymethyl)-2-

nitroimidazole), benzamide riboside, synthetic glycoamines, TNF-α, anti-Fas antibody, thapsigargin, TGF-β (TGF-β-1, TGF-β -2 or TGF-β-3), non-progestin/non-estrogen TGF-β inducing agents, polyclonal antibodies, monoclonal antibodies, dietary flavanoids, anti-inflammatory drugs, monoterpenes, S-adenosyl-L-methionine, selenium, and vitamin D compound.

Other chemotherapeutic agents include biological agents such as a p53 protein or gene, Mycobacterium phlei (M. Phlei) DNA (M-DNA) & DNA complexed with M. phlei cell wall (MCC), extract of Melothria indica Lou, fetuin, Apogen P-1a, Apogen P-1b, Apogen P-1c, Apogen P-2, and Apogen L. Further apoptotic agents include radiological apoptotic agents such as radioisotopes and DNA damaging radiation such as X-rays, UV-light, gamma-rays and microwaves.

Other therapeutic agents not listed above, but which are beneficial in combination therapies of the invention, are contemplated as within the invention.

In one embodiment, compositions of the invention and/or other agents may be administered in a single composition. However, the present invention is not so limited. In other embodiments, compositions of the present invention may be administered in one or more separate formulations from other compositions of the invention, chemotherapeutic apoptotic agents, biological apoptotic agents, radiological apoptotic agents, or other agents as is desired.

Compounds and compositions of the present invention may be formulated for administration via sterile aqueous solution or dispersion, aqueous suspension, oil emulsion, water in oil emulsion, site-specific emulsion, long-residence emulsion, sticky-emulsion, microemulsion, nanoemulsion, liposomes, microparticles, microspheres, nanospheres, nanoparticles, minipumps, and with various natural or synthetic polymers that allow for sustained release. The compounds of the present invention may also be formulated into aerosols, tablets, pills, sterile powders, suppositories, lotions, creams, ointments, pastes, gels, hydrogels, sustained-delivery devices, or other formulations used in drug delivery.

The particular formulation or formulations used will vary according to the route(s) of administration desired. For example, injectable formulations can be prepared by combining the compositions with a liquid. The liquid can be selected

from among water, glycerol, ethanol, glycols, such as propylene glycol and polyethylene glycol, oils, and mixtures thereof, and more preferably the liquid carrier is water. In one embodiment, the oil is vegetable oil. Optionally, the liquid carrier contains a suspending agent. In another embodiment, the liquid carrier is an isotonic medium and contains about 0.05% to about 5% suspending agent.

In one embodiment, the pharmaceutical composition is in unit dosage form, e.g. as tablets or capsules. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packaged powders, vials, ampoules, pre-filled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be an appropriate number of any such compositions in package form.

D. Pharmaceutical Kits

The present invention provides kits or packages of pharmaceutical formulations including the compounds or compositions described herein. The kits are also preferably organized to indicate a single oral or intravenous formulation or combination of oral formulations to be taken at each desired time, preferably including oral tablets to be taken at each of the times specified, and more preferably one oral tablet will contain each of the combined periodic dosages indicated.

The kit can also include one or more chemotherapeutic agents, biological apoptotic agents, or other therapeutic agents, such as one or more agent(s) selected from among those previously described. One of skill in the art would readily be able to formulate a suitable amount of the above-described agents for use in the kits of the invention. Kits containing radiological agents in combination with the compositions of the invention are also contemplated.

When the compounds or compositions described herein are to be delivered continuously, a package or kit can include the compound in each dosage unit (e.g. solution, lotion, tablet, pill, or other unit described above or utilized in drug delivery). When the compound is to be delivered with periodic discontinuation, a package or kit can include placebos during periods when the compound is not delivered. When

varying concentrations of a composition, of components of a composition, or of relative ratios of compounds or agents within a composition over time is desired, a package or kit may contain a sequence of dosage units, so varying.

A number of packages or kits are known in the art for the use in dispensing pharmaceutical agents for such use. Preferable, the package has indicators for each period, and more preferably is a labeled blister package, dial dispenser package, or bottle. The packaging means of a kit may itself be geared for administration, such as an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into a subject, or even applied to and mixed with the other components of the kit.

The compositions of these kits also may be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another packaging means.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of packages, the kits of the invention also may include, or be packaged with a separate instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measuring spoon, eye dropper or any such medically approved delivery means. Other instrumentation includes devices that permit the reading or monitoring of reactions *in vitro*.

In one embodiment, a pharmaceutical kit of the invention including at least one composition according to the invention in a dosage unit. In still other embodiments, pharmaceutical kits of the invention also contain chemotherapeutic apoptotic agents, biological apoptotic agents, and/or other therapeutic agents as described above. In still other embodiments, pharmaceutical kits of the invention employ the above-described compositions along with radiological agents and treatments.

E. Methods of Treatment

The modified MT-heavy metal complexes of this invention, components thereof and pharmaceutical composition containing same are useful in methods for treating cancer. Specific cancers to be treated with the compositions of the invention include estrogen negative breast cancer, estrogen positive breast cancer, prostate cancers (including androgen-independent prostate cancer), ovarian cancer, bladder cancer, brain cancer, head and neck cancer, kidney cancer, lung cancers such as small cell lung cancer and non-small cell lung cancer, myeloma, neuroblastoma/ glioblastoma, pancreatic cancer, skin cancers, liver cancers, melanoma, colon cancer, cervical carcinoma, and leukemia, retinoblastoma, pancreatic islet carcinoma, or other epithelial-derived cancers. The methods of this invention have particular applicability to those cancers which are currently treated with divalent heavy metal ions. Among such cancers are included, without limitation, brain cancer, head and neck cancers, thyroid cancer, lung cancer, ovarian cancer, prostate cancer, and bladder cancer.

Such methods include administering to a mammalian subject, preferably a human subject an effective amount of the modified MT-heavy metal complexes of this invention. Such treatment permits delivery of suitable dosages of divalent metal ions to treat the cancer, and yet inhibits the renal uptake of the heavy metals. Thus such methods have an advantage over currently employed chemotherapeutic treatments with heavy metals and are likely to permit the use of current doses more safely or higher, more effective dosages with less danger of toxicity. For example, current dosages of cisplatin for cancer therapy are between 50 to 270 mg/m^2. Because the compositions of this invention do not accumulate in the kidneys, higher doses of the heavy metals, which are more likely to be able to kill a targeted tumor or cancer cell, may be administered with less concern for toxicity to the treated patient. Methods employing these compositions inhibit renal uptake of the therapeutic divalent metal ions by megalin receptors in the proximal tubules of the kidneys. Thus, these methods also permit current dosages of heavy metals to be employed in anti-cancer therapies more safely.

In one embodiment, a method of treating or preventing the development of cancer in a mammalian subject comprising treating cancer cells of said subject with a composition of the invention is contemplated, either *in vivo* or *ex vivo*. The pharmaceutical compositions of the present invention may be administered to a subject via one or more routes to contact the cancer cells, as desired. For example, the compositions may be administered via oral, topical, systemic, enteral, parenteral (e.g., formulated for injection via the intravenous, intramuscular, subcutaneous, intracutaneous, or even intraperitoneal routes (e.g. by drip infusion)), subcutaneous, intra-portal, intra-prostatic, intra-muscular, intra-venous, intra-arterial, intra-dermal, intra-thecal, intra-lesional, intra-tumoral, intra-bladder, intra-vaginal, intra-ocular, intra-rectal, intra-pulmonary, intra-spinal, transdermal, and subdermal routes. Further, the compositions may be delivered via placement within cavities of the body, regional perfusion at the site of a tumor or other desired location, nasal inhalation, pulmonary inhalation, impression into skin and electrocorporation. The route(s) of administration will vary according to the cell(s), tissue(s), organ(s), or system(s) to be treated.

In a further embodiment, the compounds are delivered transdermally or by sustained release through the use of a transdermal patch containing the composition and an optional carrier that is inert to the compound, is nontoxic to the skin, and allows for delivery of the compound for systemic absorption into the blood stream. Such a carrier can be a cream, ointment, paste, gel, or occlusive device. The creams and ointments can be viscous liquid or semisolid emulsions. Pastes include absorptive powders dispersed in petroleum or hydrophilic petroleum. Further, a variety of occlusive devices can be utilized to release the active reagents into the blood stream and include semi-permeable membranes covering a reservoir contain the active reagents, or a matrix containing the reactive reagents.

The use of sustained delivery devices can be desirable, in order to avoid the necessity for the patient to take medications on a daily basis. The term "sustained delivery" is used herein to refer to delaying the release of an active agent, i.e., a compound of the invention, until after placement in a delivery environment, followed by a sustained release of the agent at a later time. A number of sustained delivery

devices are known in the art and include hydrogels (US Patent Nos. 5,266,325; 4,959,217; 5,292,515), osmotic pumps (US Patent Nos. 4,295,987 and 5,273,752 and European Patent No. 314,206, among others); hydrophobic membrane materials, such as ethylenemethacrylate (EMA) and ethylenevinylacetate (EVA); bioresorbable polymer systems (International Patent Publication No. WO 98/44964 and US Patent Nos. 5,756,127 and 5,854,388); and other bioresorbable implant devices composed of, for example, polyesters, polyanhydrides, or lactic acid/glycolic acid copolymers (US Patent No. 5,817,343). For use in such sustained delivery devices, the compounds of the invention can be formulated as described herein. See, US Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; and 4,008,719.

The methods of this invention involve administering such compositions in effective amounts to induce apoptosis in the cancer cells, while minimizing adverse impacts on non-cancer cells of the patient. Dosages of the compounds and compositions of the present invention vary with the particular compositions employed, the route of administration, the severity of the symptoms presented, the particular subject being treated, and the subjects other medications and treatment, as well as the subject's medical history. Precise dosages for intravenous, oral, parenteral, nasal, or intrabronchial administration can be determined by the administering physician based on experience with the individual subject treated. An effective therapeutic dosage will contain a dosage sufficient to induce apoptosis of cancer cells.

The amount of the compound of the invention present in each effective dose is selected with regard to consideration to the half-life of the compound, the identity and/or stage of the cancer, the patient's age, weight, sex, general physical condition and the like. The amount of active component or compound required to induce an effective apoptotic effect on cancer cells without significant adverse side effects varies depending upon the pharmaceutical composition employed and the optional presence of other components. Suitable dosages of compositions used to treat cancers as described herein can range from 1.0 μg to 500 mg MT sequence(s)/kg patient body weight. In one embodiment, the dosage is at least 10 μg/kg. In another embodiment, the dosage is at least 100 μg/kg. In another embodiment, the dosage is at least 500

μg/kg. In another embodiment, the dosage is at least 1 mg/kg. In another embodiment, the dosage is at least 10 mg/kg. In another embodiment, the dosage is at least 50 mg/kg. In another embodiment, the dosage is at least 100 mg/kg. In another embodiment, the dosage is at least 250 mg/kg. In another embodiment, the dosage is at least 400 mg/kg. In another embodiment, the dosage is at least 500 mg/kg. In another embodiment, each dose will contain between about 5 μg peptide/kg patient body weight to about 10 mg/kg. Generally, a useful therapeutic dosage is between 1 to 5 mg peptide/kg body weight. Another embodiment of a useful dosage may be about 500 μg/kg of peptide. Other dosage ranges may also be contemplated by one of skill in the art. For example, dosages of the peptides of this invention may be similar to the dosages discussed for other cancer therapeutics. As one embodiment, the dosages may be provided in terms of mg heavy metal per meter square. For example, the amount of the MT-heavy metal complex to be delivered may be greater than 50 mg/m^2 and possibly greater than $100mg/m^2$ or greater than 270 mg/m^2. Initial doses of a composition of this invention may be optionally followed by repeated administration for a duration selected by the attending physician. Dosage frequency may also depend upon the factors identified above, and may range from 1 to 6 doses per day for a duration of about 3 days to a maximum of no more than about 1 week. The compositions of this invention may also be administered as a continuous infusion for about 3-5 days, the specific dosage of the infusion depending upon the half-life of the compound. The compounds of this invention may also be incorporated into chemotherapy protocols, involving repetitive cycles of dosing. Selection of the appropriate dosing method would be made by the attending physician.

In another embodiment of this invention, the method of treating or preventing the development of cancer in a mammalian subject involves exposing the subject to one or multiple (e.g. 2, 3, 4, or more) chemotherapeutic apoptotic agents, biological apoptotic agents, radiological apoptotic agents, or other therapeutic agents described herein. Such combination treatment may occur by administering compositions containing multiple active ingredients, as described above. However, this invention also encompasses a method of administration of anti-cancer agents or therapies in conjunction with a composition containing a modified MT-heavy metal ion complex

as described above. In one embodiment, a different chemotherapeutic agent is administered before treatment with a composition of the invention. In another embodiment, a different chemotherapeutic agent is administered after treatment with a composition of the invention. In still another embodiment, a chemotherapeutic or biological apoptotic agent is administered before, during or after treatment with a composition of the invention.

In still another embodiment, the patient is exposed to radiological treatment administered before, during or after treatment with a composition of the invention. Where a radiological agent is desired in combination with one or more of the compounds or compositions of the present invention, dosage may be determined by an administering physician according to standard regimens taking into account other factors including other treatments applied in combination. For example, the appropriate regimen of radiation dosage ranges for X-rays ranges from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by cells.

The compositions of this invention may also be employed in a method for treating heavy metal poisoning in a mammal, preferably a human. A modified MT sequence or fragment as described above, e.g., the hinge region sequence SCKKSCC itself, is administered to the subject in an amount sufficient to compete *in vivo* with naturally circulating MT for complexation with any heavy metal in the circulation. Excess amounts of the modified MT may be employed to scavenge any heavy metal in the circulation. The modified MT-heavy metal complexes are then eliminated from the body by bypassing the megalin receptors in the kidneys. Suitable dosages of the modified MT sequences can be determined by an attending physician based upon the condition of the patient, the heavy metal involved, the level of poisoning, etc.

The identification of megalin as the receptor for MT binding in the renal tubules also permits a method for the treatment of heavy metal poisoning by treating a patient known to have ingested or to have been exposed to heavy metals with an excess amount of a known megalin ligand. Many such ligands are known in the art,

as evidenced by the publications cited herein, as is the hinge region itself. By treating the patient with an excess of a suitable megalin ligand, particularly a ligand that binds megalin with an avidity greater than that of naturally-occuring MT, megalin receptors in the kidneys can be bound by such ligands, thereby reducing the number of receptors available to bind the circulating MT that forms complexes with the heavy metal in the patient's circulatory system. In this manner, the naturally-formed complexes can be excreted without opportunity to bind in the renal tubules. Thus, a patient so treated, may eliminate more of the heavy metal and experience less toxicity. Suitable dosages of the megalin ligands can be determined by an attending physician based upon the condition of the patient, the heavy metal involved, the level of poisoning, etc. However, such dosages are likely to be quite high, perhaps over 100 times the amount of naturally-occurring MT.

F. Method for Developing Novel Therapeutics for Treatment of Cancer or Heavy Metal Poisoning.

The present invention and the discovery of the megalin receptor of MT provides yet another aspect of this invention, i.e., a method for identifying a test compound for cancer treatment. For example, a battery of new or known compounds is assayed for the ability to compete with naturally-occurring MT for binding to the megalin receptor. Compounds that bind megalin more avidly than MT may be useful for the treatment of heavy metal poisoning, as described above. In contrast, compounds that bind megalin less avidly than MT and can be engineered to also bind therapeutic metal ions may be useful as cancer therapeutics, in the same manner as described above for the modified MT sequences.

Thus, in one embodiment, a method for identifying therapeutic compounds includes contacting *in vitro* a sample of immobilized megalin receptor with a test compound and naturally-occurring MT. Once any unbound substances are eliminated, the relative amounts of MT and the test compound which are immobilized on the megalin receptor are measured. The presence of excess test compound indicates a compound that may be useful for the treatment of heavy metal poisoning. An excess of bound MT indicates that the test compound binds megalin less avidly

than MT and, if the test compound is also capable, or may be made capable of binding heavy metal ions, the test compound may be useful to deliver such ions *in vivo* for cancer treatment.

Other assay protocols known to the art may be designed which employ binding to megalin as the critical determination for identification of a suitable compound for use as a drug for this purpose.

G. Examples

The following examples illustrate various aspects of this invention. Examples 1-6 provide evidence indicating that megalin binds MT and is the receptor responsible for the uptake of Cd-MT in the proximal convoluted tubules. Megalin is by far the most quantitatively important mechanism of MT uptake into the renal proximal tubule. First, SPR directly demonstrates binding of the purified proteins in a dose-, ion-, and pH-dependent manner. Second, antibody interference experiments show that >90% of the MT binding on brush-border membrane vesicles, and cellular uptake into BN-16 cells, can be displaced or inhibited specifically with anti-megalin, but not control, antisera. Finally, megalin and MT colocalize at the cellular level in fluorescent microscopy studies. Megalin and MT colocalize and internalize concomitantly before separating in the late endosomal pathway. The Examples 7 - 8 demonstrate that a reagent comprising a mutant MT that does not bind megalin, but binds heavy metals, is taken up by cancer cells and maintains clinical efficacy.

These examples do not limit the scope of this invention which is defined by the appended claims. One skilled in the art will appreciate that although specific reagents and conditions are outlined in the following examples, modifications can be made which are intended to be encompassed by the spirit and scope of the invention.

EXAMPLE 1: ANIMALS, REAGENTS, ANTIBODIES, STATISTICS

Male Sprague-Dawley rats (200–250 g) were obtained from Sasco (Omaha, NE). All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

These studies used commercially available MT-I, isolated from either rabbit liver or horse kidney, which is a highly conserved mammalian isoform. All known

class I sequences contain 61 or 62 amino acids with 20 conserved cysteine residues and are able to bind up to 7 equivalents of divalent metal ions, commonly a mixture of zinc and cadmium. Although Zn-MT and Cd-MT differ dramatically in their toxic effects, they produce virtually identical profiles in their binding and uptake (Dorian C and Klaassen CD. 1995 *Fundam Appl Toxicol* 26: 99–106). In solution, MT tends to form oligomers (Tang W et al, 1999 J Anal Toxicol 23: 153–158). Therefore, MT was used as received, rather than saturated with cadmium in an extra step, to maximize the structural integrity of the metalloprotein during our analyses. The inclusion of recombinantly expressed MT samples in these examples is an important control for any impurities in the commercial reagents. The supplier-reported metal assays of MT samples show ~7% metals by mass, which indicated complete occupation of all metal-binding sites by zinc and/or cadmium.

In preliminary experiments, the inventors found that DTT, which is frequently used as a reducing agent in solution with MT to prevent oxidation of MT, potently denatures megalin, abolishing the binding of known megalin ligands. Thus the inventors continually used an internal control having cysteine content similar to active peptides to correct for the nonspecific effects of sulfydryl binding. The ability of the MT-fragment *peptide 2* (aa 10-25 of SEQ ID NO: 1) partially to inhibit binding of MT to brush-border membranes, but not uptake in BN-16 cells, falls into this category.

Purified human megalin and cubilin receptors were obtained by detergent solubilization of renal cortex brush-border membranes followed by affinity chromatography using immobilized receptor-associated protein (Moestrup SK. et al, 1998 *J Biol Chem* 273: 5235– 5242). Polyclonal antibodies against cubilin, megalin, and transferrin were raised against proteins purified by immunoaffinity chromatography using previously reported monoclonal antibodies coupled to Sepharose 4B (Hammond TG et al, 1994 *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F516–F527; Moestrup, cited above, Sahali D et al, 1988 *J Exp Med* 167: 213–218; Sahali D et al, 1993 *Am J Pathol* 142: 1654–1667). These antibodies were monospecific by immunoblotting on whole brush-border preparations and by immunoprecipitation of biosynthetically labeled yolk sac epithelial cells in culture.

Among the antibodies employed were: anti-neurokinin-1 (NK1)/substance-P receptor antiserum (Dr. Jacques Couraud, Gif-sur-Yvette, France) (Bret-Dibat JL, et al., 1994 *J Neurochem* 63: 333–343); anti-giantin (Dr. H. P. Hauri, University of Basel). Antibodies to angiotensin II type 1 (AT1) receptor (Santa Cruz Biotechnology, Santa Cruz, CA).; fluorescent secondary antibodies: mouse FITC-anti-goat antibody (Dako, Carpinteria, CA) or goat anti-mouse, goat anti-rabbit, and donkey anti-sheep antibodies, all conjugated to Alexa 488 (Molecular Probes, Eugene, OR).

Synthetic peptides corresponding to portions of the MT sequence (FIG. 1) were obtained from Biosource International, Camarillo, CA). Recombinant production of full-length MT (rMT fl) and the two individual domains (rMT-α and rMT-β) were prepared as described in Atrian S, et al., "Recombinant synthesis and metal-binding abilities of mouse metallothionein 1 and its α-and β-domains." In: *Metallothionein IV*, ed. Klaassen CD. Basel: Birkha"user Verlag, 1999, p. 55–61. The protein yields and atomic absorption validation of metal content of the recombinant proteins are shown in Table 1.

TABLE 1: Protein yield and heavy metal content of recombinant mouse MT proteins

Clone	Protein Concentration/ Total Yield by A_{280}	Zn Content as Zn/Protein Ratio by inductively coupled plasma	Predicted Ideal Zn/Protein Ratio
rMT fl	$1.18x10^{-4}$ M/2.14 mg total	6.73	7
rMT-α	$1.37x10^{-4}$ M/1.58 mg total	3.93	4
rMT-β	$1.87x10^{-4}$ M/2.01 mg total	3.01	3

Data in the following examples are expressed as means ± SE throughout the manuscript. Statistical analysis was performed by analysis of variance and Bonferroni or Scheffe''s post hoc comparison. Flow cytometry data were also analyzed by Kolgomorov-Smirnov summation statistics (Young IT. 1977 *J Histochem Cytochem* 25: 935–941).

EXAMPLE 2: MOLECULAR STUDIES OF RECEPTOR-MT BINDING USING SPR.

Cubilin and megalin were studied separately by using SPR, immobilizing purified membrane-free samples of each receptor, and studying its interaction with rabbit liver MT. The interaction of MT with megalin was assayed with a BIACORE 3000 biosensor system (Biacore). In surface plasmon resonance (SPR), one protein is immobilized to a dextran-coated gold surface. Injection of a soluble protein produces a signal change that is directly proportional to the mass of bound protein and is reported as resonance units (RU). Megalin (0.025 mg/ml in 10 mM acetate, pH 4.53) was immobilized (1,000–3,000 RU) in one flow cell on a CM5 biosensor chip using standard primary amine-coupling methods as detailed by the manufacturer. An equal amount of either ovalbumin or casein was immobilized in a second flow cell to provide real-time reference correction for instrumental artifacts and nonspecific binding events.

Rabbit liver MT was injected over both flow cells at room temperature in HEPES-buffered saline (HBS), pH 7.4, containing 2 mM Ca, 2 mM Mg, and 0.005% surfactant P20. Maximum reproducibility was obtained when 0.0008% sodium dextran sulfate (catalog no. 17–0340-01, Pharmacia Biotech) was also included in the buffer. Equilibrium dissociation constants (Kd) were determined from steady-state binding measurements at concentrations of 75, 150, 300, 600, 1,200, and 2,400 µg/ml MT. Proteins were typically injected at flow rates of 50 µl/min for 3 min and then allowed to dissociate for 5 min.

Because MT is a low-affinity ligand, no regeneration (removal of bound protein by injection of a second, typically harsh, solvent) was necessary. The "double-referencing" technique (Myszka, cited above) was used to eliminate additional instrumental artifacts. The blank injections used for this procedure were identical to sample solutions except for the omission of MT. Thermodynamic constants were calculated using Biacore's BIAevaluation 3.1 software.

The dose-dependent binding to megalin (not shown) uniformly increased with dose over a 32-fold increase in concentration, 75–2,400 µg/ml. The observed variations and noise were normal for the very low signal levels used. The fit of

maximum responses obtained after 2.5 min. is illustrated in the graph of FIG. 2. Even at high MT concentrations, >90% saturation was not achieved, and therefore some errors occurred in the fit. An approximate fit using the maximum (but non equilibrium) responses obtained at each concentration yielded an estimated dissociation constant of 9.8×10^{-5} M (FIG. 2). Repeated experiments consistently indicate the binding of ~0.7–0.9 mol of MT/mol of megalin, consistent with one binding site. In contrast, no binding of MT to cubilin was observed.

The binding shown in FIG. 2 was specific for megalin and depended on metal ions but not on the MT source. Omitting either Ca or Mg from the sample buffers abolished the binding (data not shown); both appeared to be required. Samples of MT from horse kidney and from rabbit liver provided nearly identical results (data not shown). Interestingly, oligomerized MT bound more effectively to megalin than did the monomer. Nondenaturing gel electrophoresis showed that over time, MT forms trimers, tetramers, and even much larger oligomers (data not shown). The binding of such molecules to megalin was significantly stronger. Owing to difficulties in purifying these oligomers, the actual binding constants for oligomers could not be determined with any precision. Qualitatively, compared with monomeric MT, oligomeric MT dissociated much more slowly, and harsher conditions were required to dislodge it from immobilized megalin. Using the tetramer as a basis for calculations, one may estimate a 100-fold change in Kd (7×10^{-7} M).

EXAMPLE 3: INHIBITION OF MT BINDING BY MEGALIN LIGANDS AND SMALL PEPTIDES DERIVED FROM MT.

A. Peptides

A series of peptides spanning the sequence of rabbit liver MT were prepred and used SPR to study their effect on the binding of MT to megalin (see FIG. 1). Six 16-amino acid peptides, spanning the entire MT sequence with each overlapping its neighbors by 7 amino acids (Biosource International; Kägi JHR and Vasak M., "Chemistry of mammalian metallothionein." In: *Metallothionein in Biology and Medicine*, eds. Klaassen CD and Suzuki KT., Boca Raton, FL: CRC, 1991, p. 49–60) were used to inhibit the binding of MT to megalin. The SCKKSCC

peptide (aa 28-34 of SEQ ID NO: 1)represented the overlap sequence between two of these peptides (Biosource International). The peptide concentrations were ~5 μM, while rabbit liver MT was ~250 μg/ml, corresponding to the ligand *K*d (estimated).

B. Preparation of recombinant mouse MT and α- and β-subunit

Recombinant fragments of mouse MT were produced successfully and reproducibly (Atrian et al., cited above) by making recombinant MT subunits in *Escherichia coli* using a GST fusion vector followed by thrombin cleavage to release the free MT subunit. This approach is more practical than site-directed mutagenesis, as most attempts to produce recombinant MT have been characterized by very low yields or by mixtures of several short cleavage fragments of the MT molecule (Huang PC et al, "Native and engineered metallothioneins." In: *Metallothionein in Biology and Medicine*, ed. Klaassen CD and Suzuki KT., Boca Raton, FL: CRC, 1991, p. 87–101). The thrombin cleavage leaves three amino acids, specifically SCM derived from the COOH terminus of the GST, on the NH_2 terminus of the product.

To understand the data, the inventors postulated the critical binding site on MT to be the intradomain SCKKSCC (aa 28-34 of SEQ ID NO: 1) region, with SCK representing the COOH-terminal end of the β-subunit and KSCC the NH_2-terminal start of the α-subunit. The recombinant α-subunit, therefore, has a conservative GST-derived SCM substitution for SCK on its NH_2 terminus, leaving the postulated critical SCKKSCC sequence (aa 28-34 of SEQ ID NO: 1) essentially intact. The recombinant β-subunit starts with SCM-and ends in SCK, rendering the postulated critical SCKKSCC (aa 28-34 of SEQ ID NO: 1) disrupted. The full-length recombinant MT has an intact SCKKSCC (aa 28-34 of SEQ ID NO: 1) sequence as well as an additional NH_2-terminal SCM. Atomic absorption (inductively coupled plasma) analysis of the zinc content of the recombinant subunits proved them to be at the predicted heavy metal content to within the error of the methods (see Table 1).

C. SPR analysis of binding.

Megalin was immobilized as described above in Example 2. An equal amount of transferrin (0.10 μg/ml in 10 mM acetate, pH 4.96) was immobilized in a second flow cell to provide real-time reference correction. Dose-dependent peptide binding was examined by injecting the peptide at concentrations ranging from 0 to 500 μg/ml.

Inhibition of MT binding by peptide was examined by injecting rabbit liver MT as described above and comparing the results to samples that contained varying concentrations of peptide but were otherwise identical. No regeneration was necessary. Additional artifacts were eliminated before curve fitting by applying double-referencing techniques. The blank injections used for this procedure were identical to sample solutions except for the omission of MT and peptide.

Peptides unrelated to MT but having a central KK motif, specifically a v-ATPase β-subunit peptide with a KK motif (CLQKFEKKINQSPYEKR; SEQ ID NO: 5) and an apolipoprotein A-I peptide with KK motif (ALEEYTKKLNTQ; SEQ ID NO: 6; Biosource International), served as control peptides.

Protein concentrations were assayed by the Bradford method (Pierce Biotechnology, Rockford, IL). Recombinant mouse MT proteins, and native mouse MT as a control, were dialyzed into SPR binding buffer with magnesium and calcium under acidic conditions to remove the zinc. Aliquots of the proteins were reconstituted with zinc by simple alkalinization in the presence of the metal, and excess metal was removed with resin. The proteins were used for SPR analysis or redialyzed into appropriate buffers for cell uptake studies.

Results obtained by injecting synthetic peptides in HEPES-buffered saline (HBS) containing 2 mM Ca and Mg in these initial qualitative SPR studies are summarized in Table 2.

TABLE 2: Binding to megalin by peptides derived from MT and interference with binding of the native protein using SPR techniques

Peptide Fragments	Sequence	Binding to Megalin	Competition with MT for Megalin Binding
1 (SEQ ID NO: 7)	KMDPNCSCATGNSCTCA	No	No
2 (aa10-25 of SEQ ID NO: 1)	GNSCTCASSCKCKECK	No	No
3 (aa19-34 of SEQ ID NO: 1)	CKCKECKCTSCKKSCC	Yes	Yes
4 (aa28-43 of SEQ	SCKKSCCSCCPAGCTK	Yes	Yes

ID NO: 1)			
5 (aa37-52 of SEQ ID NO: 1)	CPAGCTKCAQGCICKG	Insoluble; no data	Insoluble; no data
6 (aa44-61 of SEQ ID NO: 1)	CAQGCICKGASDKCSCCA	No	No

When using SPR, no reproducible inhibition of MT binding to megalin by known megalin ligands was observed, but inhibition by some of the synthetic peptides corresponding to sequences within MT was noted. Commercial sources of β2-microglobulin dissociated only with difficulty from the immobilized megalin, leading to erratic, nonreproducible binding, and loss of binding of control ligands after the harsh regeneration modalities necessary. For this reason, SPR assessment of competitive β2-microglobulin binding with MT was impractical.

Interestingly, peptides 3 and 4 bound quite tightly to megalin and also disrupted the binding of MT. Although peptides 1, 2, and 6 contain cysteines, they did not bind megalin, suggesting that the binding of peptides 3 and 4 is a specific interaction, rather than a nonspecific disulfide interaction between the peptides and megalin. Technical issues prevented direct confirmation; reduction with DTT denatured megalin and abolished the binding of all ligands. Because the behavior of peptides 3 and 4 differed significantly from that of the other soluble peptides, attention focused on the overlap sequence these peptides have in common.

SPR analysis of the binding of hinge peptide SCKKSCC (aa 28-34 of SEQ ID NO: 1) in HBS containing 2 mM Ca and Mg and altered binding of MT to megalin in the presence of the peptide was assayed using SPR techniques of Example 2. In the resulting graph (not shown) traces represented the responses obtained with 63, 125, 250, and 500 μg/ml peptide. Each trace represented the average of 3 replicates and was corrected by referencing to blank buffer injections. Responses were also obtained when MT was injected alone and in the presence of hinge peptide SCKKSCC (aa 28-34 of SEQ ID NO: 1). The response when MT is injected at a concentration of 2,000 μg/ml and the response when MT (2,000 μg/ml) and peptide

(250 μg/ml) are coinjected were plotted on a graph (not shown). Each trace represents the average of 3 replicates and was corrected by referencing to blank buffer injections.

The results indicated that a peptide representing this overlap sequence, SCKKSCC (aa 28-34 of SEQ ID NO: 1), bound to megalin and also disrupted the binding of native MT (see, also, Table 3). The dose-dependent binding of this peptide to megalin is shown in Table 3.

The ability of the peptide to affect the binding of MT to megalin is apparent in which the binding of MT decreased when coinjected with peptide. In contrast, peptides containing a lysine repeat but derived from unrelated ATPase or apolipoprotein A-I sequences had no apparent effect, producing instead responses that were essentially additive (Table 3). The polybasic megalin ligand, gentamicin, bound megalin with an affinity much lower than MT and showed no interference with MT binding (Table 3).

SPR analysis of binding to megalin shows that, when corrected for the molecular mass of the protein fragments ($n = 2$ for each analysis), the recombinant full-length MT clone bound 95% as well as the native protein, the α-subunit with an intact conservatively substituted SCKKSCC (aa 28-34 of SEQ ID NO: 1) region, also bound ~94% as well as the native MT (see Table 4). However, the β-subunit in which the SCKKSCC (aa 28-34 of SEQ ID NO: 1) region is divided at KK has binding reduced to 30% of the predicted value (see Table 4).

TABLE 3: Binding to megalin by polybasic peptides and gentamicin and interference with binding of native MT using surface plasmon resonance techniques

Peptide or Reagent	Sequence	Binding to Megalin Compared With MT	Effect on MT Binding to Megalin
MT sequence overlap of *peptides* 3 and 4*	SCKKSCC (aa 28-34 of SEQ ID NO: 1)	Affinity ~ MT	Competitive with MT binding
v-ATPase β-subunit peptide with KK motif	CLQKFEKKINQSPY EKR (SEQ ID NO: 5)	Affinity << MT	Additive to MT binding
Apolipoprotein-A-1 peptide with KK motif	ALEEYTKKLNTQ (SEQ ID NO: 6)	Affinity << MT	Additive to MT binding
Gentamicin		Affinity <<< MT	Additive to MT binding

TABLE 4: Binding to megalin by recombinant proteins derived from mouse MT and interference with binding of the native protein using SPR techniques

Clone	% Predicted Binding by SPR
Native full-length MT	100 (positive control)
MT full-length recombinant	95
MT α-subunit recombinant	94
MT β-subunit recombinant	30

EXAMPLE 4: PROTEIN-RECEPTOR BINDING IN MEMBRANE VESICLES AND DISPLACEMENT BY ANTIBODIES STUDIED BY FLOW CYTOMETRY.

A. Preparation f Fluorophore-Conjugated MT.

MT was conjugated to Alexa Fluor 594, FluorX, or Cy3 (Molecular Probes) following the supplier's protocols. Because MT is a very small protein, unreacted dye was removed by dialysis against PBS at pH 7.4 in Slide-A-Lyzer dialysis cassettes having 3,500-kDa molecular mass cutoff (catalog no. 66330, Pierce) rather than with the use of the columns provided in the manufacturer's kit.

B. Flow cytometry analysis of the displacement of fluorescently labeled MT from brush-border membranes by anti-receptor antisera, peptides and ligands.

Rat renal cortical brush-border membrane vesicles were isolated by magnesium precipitation techniques as described previously (Batuman V, et al., 1990 *Am J Physiol Renal Fluid Electrolyte Physiol* 258: F1259–F1265; Hammond TG et al. 1994 *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F516–F527; Sahali, cited above.) and incubated with fluorescently conjugated MT and receptor antisera.. The binding of MT was investigated in the presence of 100-to 3,300-fold dilutions of anti-cubilin or anti-megalin polyclonal antibodies that recognize the holoprotein. Antibodies to the AT_1 receptor and anti-NK_1 peptide antibodies were chosen as negative controls for nonspecific interference by binding because they bind brush-border membrane vesicles at the same titer as the anti-megalin antisera.

Binding of FluorX (Amersham Biosciences, Piscataway, NJ)-conjugated MT was analyzed by flow cytometry using a FACStar Plus flow cytometer (Becton Dickinson Immunocytochemistry, San Jose, CA) to collect data files of 2,000 observations/sample. All antisera were used at 1:1,000 dilutions, which represented peak binding on dilution curves. Synthetic peptides were used at concentrations of 400μg/ml, which was enough to inhibit significantly the binding of MT when observed by SPR. For consistent comparison, the known megalin ligand β2-microglobulin was also used at 400μg/ml.

C. Results

The binding of fluorescent MT to vesicles was readily detected (FIG. 3). The observed fluorescence is shown for the control (MT alone) and MT in the presence of anti-cubilin, anti-megalin, and anti-NK1-peptide antibodies. Data files of 2,000 observations/sample were collected. The addition of anti-megalin antibodies was able to displace nearly all bound MT (no antibody, MT binding 161 ± 4 fluorescence units, $n = 5$; anti-megalin antibodies 14 ± 5, $n=5$, $P<0.01$ by ANOVA and Scheffé, as well as on each individual run by Kolgomorov-Smirnov, cited previously. Antibodies to cubilin had a small but significant effect on binding (146 ± 5, $n = 5$, $P < 0.05$ by Kolgomorov-Smirnov compared with no antibody). However, antiserum to the

unrelated NK1 receptor (a negative control) had little or no effect (150 ±21, $n = 5$, $P >$ 0.05 compared with no antibody).

The binding of fluorescent MT to freshly prepared rat brush-border membrane vesicles was readily detected [3±1 arbitrary fluorescence units in unstained control vesicles, compared with 534 ± 180 when vesicles were labeled with fluorescent MT, n = 6, means ± SD] (FIG. 4). The addition of *peptide 2* (amino acids 10–25 of SEQ ID NO: 1), at equimolar concentrations to the fluorescent MT reduced MT binding to 434 ±156 ($P < 0.05$ by ANOVA and Scheffé as well as on each individual run by Kolgomorov-Smirnov) (Young IT. 1977 *J Histochem Cytochem* 25: 935–941), with further reduction to 336 ± 97 when fluorescent MT competed with equimolar overlap peptide SCKKSCC (aa 28–34 of SEQ ID NO: 1; $P < 0.01$). The known megalin ligand β2-microglobulin, which has been demonstrated to compete with MT in live rat studies (Bernard A, et al, 1988 *Kidney Int* 34: 175–185), competed with fluorescent MT at equimolar concentrations (394 ± 128, $n = 6$, $P < 0.05$), whereas antibodies to the unrelated AT1 receptor (a nonspecific control) had little or no effect (513 ± 157). Fluorescently conjugated MT competes directly with equimolar unlableled MT (398 ±114, $n = 6$, $P < 0.05$ compared with MT, no competition). Solubility limitations prevented study of higher competing concentrations of peptides.

EXAMPLE 5: CELL CULTURE STUDIES BY FLUORESCENCE MICROSCOPY

A. Preparation of Cell Cultures

Except as noted, experiments were conducted using immortalized yolk sac cells from the Brown Norway rat (BN16) (Le Panse S. et al, 1997 *Exp Nephrol* 5: 375–383). An apical brush border and a specialized endosomal pathway similar to the renal proximal tubule, including abundant expression of megalin and cubilin, characterize these cells. The cells were grown in DMEM (GIBCO/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 50 μg/ml streptomycin or ciprofloxacin. Cells were passaged every 4 days with a split ratio of 10:1. Madin-Darby canine kidney (MDCK) cells were grown in a modified minimal essential medium as described in American Type Culture Collection (Manassas, VA) protocols.

B. *MT Uptake by BN-16 Cells Analyzed By Epifluorescence And Confocal Microscopy.*

Uptake experiments were performed with confluent monolayers cultured in eight-chamber glass slides (Nalge Nunc, Naperville, IL). The BN-16 cells were cultured on chambered slides until confluent (~10–18 h). The monolayers were washed twice with cold PBS and allowed to equilibrate at 4°C in a cold room. The labeled MT in DMEM containing 0.01% ovalbumin was added at concentrations ranging from 0.075 to 12μM. After incubation at 37°C for 20 min, the medium was removed and the cells were washed successively with PBS/0.1% ovalbumin (2X) and PBS before being fixed and mounted. The slides were examined by use of a fluorescence microscope (Leica DMR, Basel, Switzerland) equipped with a color video camera (Sony 3CCD). This experiment was used to select a concentration of 1.0μM for subsequent experiments involving the labeled ligand.

In a time-dependent uptake experiment using labeled MT, cells were prepared as before but incubated with 1.0 μM ligand for intervals of 5, 15, 30, and 45 min. In receptor colocalization experiments, the cells were permeabilized with Triton X-100 (0.05% in PBS) and treated with the appropriate primary and secondary antibodies after fixation. The primary antibodies included anti-megalin, anti-cubilin, anti-TfR, and anti-giantin. To follow the internalization of MT, Alexa-labeled MT was added at concentrations of 1.0 or 6.0μM and the cells were incubated in the cold for intervals ranging from 5 to 45 min before being fixed.

Based on these experiments, confluent monolayers were washed with PBS and allowed to equilibrate in a cold room with labeled MT (2.5 μM) for1h at 4°C. After being washed with PBS, the cells were treated with warm DMEM containing 2.5 μM unlabeled MT and 0.01% ovalbumin and immediately transferred to an incubator. Cells were fixed at intervals of 5, 15, and 45 min. Finally, the cells were permeabilized and incubated with the appropriate primary and secondary antibodies to localize megalin, cubilin, and TfR.

C. *MT Uptake By MDCK Cells Analyzed By Confocal Microscopy.*

MDCK cells were cultured on chambered slides until confluent (~2 days). The monolayers were washed twice with PBS and treated with labeled MT in DMEM

containing 0.01% ovalbumin. The labeled MT was added at a concentration of 1.0 μM. After incubation at 37°C for 30 min, the medium was removed and the cells were washed successively with PBS/0.1% ovalbumin (2x) and PBS before being fixed and mounted. To assist in visualization of the cells, some samples were permeabilized with Triton X-100 (0.05% in PBS) and stained with DAPI. After a preliminary examination with a fluorescence microscope as described above, confocal microscopy was carried out with a Leica TCS equipped with a DMR inverted microscope and a 63/1.4 objective. Image processing was performed with the use of the Leica's online Scanware software. Numeric images were processed with the use of Scion Image and Photoshop 5.0 software.

D. MT Uptake By BN-16 Cells Analyzed By Flow Cytometry.

Uptake experiments used FluorX-or Cy3-labeled MT and were performed with confluent monolayers cultured in 96-well plates. In preliminary experiments, MT uptake was determined to be linear for at least 3 h and exhibited dose-dependent saturation. The concentration producing half-maximal uptake was ~5 μM. Inhibition experiments were performed as follows. The confluent monolayers were washed with serum-free DMEM and allowed to equilibrate for 2h at 37°C. The cells were then incubated with 5 μM labeled MT and any inhibitor for 1–2.5h at 37°C. Incubations were performed in DMEM containing 0.1% ovalbumin to reduce nonspecific binding. The cells were washed several times with PBS, acid-washed to release membrane-bound proteins, released with trypsin, and washed several more times with PBS. In this state they could be analyzed immediately, without fixing, by flow cytometry analysis. The positive control was labeled MT without added inhibitor; the negative control was unlabeled MT. Inhibitor concentrations were generally 10–100x greater than the concentration of labeled MT.

E. Results

Epifluorescence and confocal microscopy analysis of the time-dependent uptake of fluorescently labeled MT in BN-16 cells and colocalization with megalin and cubilin were performed as follows. Confluent monolayers of BN-16 cells were incubated with fluorescently labeled MT at 37°C. After 30 min, fluorescence microscopy revealed that much MT could be found in the cells in a granular form,

consistent with MT uptake into endosomes. To follow cellular uptake more closely, BN-16 cells were incubated with labeled MT at 4°C and chased with unlabeled MT for variable intervals. At 4°C, MT bound to the surface but did not enter the cells, whereas incubation at 37°C afterward led to uptake. Colocalization with antibodies to the transferrin receptor, an early endosomal marker, indicated that MT entered the early endosomes within 15 min but passed beyond them in <45 min.

Colocalization of MT with both megalin and cubilin was demonstrated by using receptor antibodies in conjunction with a fluorescent secondary antibody. At 4°C, megalin, cubilin, and MT were colocalized on the surface, whereas after 15 min at 37°C they had all migrated to the early endosomes (data not shown). After 45 min, little evidence for colocalization remained (data not shown). No colocalization was observed with antibodies to giantin, an unrelated protein found in the Golgi apparatus and used as a negative control (data not shown).

Using confocal microscopy, results with this higher resolution method for colocalization confirmed that MT and megalin or cubilin were colocalized. Similar patterns of distribution are observed for fluorescent MT and antibody (data not shown). As a negative control, MDCK cells were examined for evidence of MT uptake. These cells do not express cubilin or megalin, and in fact we found that they did not import MT at all, demonstrating that ordinary membrane diffusion (of free dye or of conjugated MT) cannot explain the results with BN-16 cells.

EXAMPLE 6: :MT UPTAKE IN CULTURED CELLS AND INHIBITION BY ANTIBODIES, LIGANDS, AND PEPTIDES STUDIED BY FLOW CYTOMETRY.

Consistent with a receptor-mediated process, MT uptake may be saturated, inhibited by receptor ligands and by MT model compounds, and inhibited by receptor antibodies. To determine the cellular uptake of fluorescently labeled MT and inhibition by known megalin ligands, we began with dose-and time-dependent uptake studies. Incubation of BN-16 cells with 0–80 μM fluorescently labeled MT for 3 h, followed by flow cytometry analysis, demonstrates that MT uptake is saturable and that MT concentrations of 4–5 μM produce half-maximal uptake. Under these conditions, the uptake of labeled MT was easily distinguished against background

signal (FIG. 6*A*). Uptake of MT by BN-16 cells was then demonstrated to be linearly time dependent at doses above and below the half-maximal binding concentration (FIG. 6*B*). The addition of β2-microglobulin reduced MT uptake in a dose-dependent manner across a broad range of concentrations of both MT and β2-microglobulin (FIG. 6*C*).

Incubating BN-16 cells with anti-megalin antibodies before adding fluorescent MT greatly reduced the uptake of MT in a dose-dependent manner (FIG. 7*A*) (unstained cells geometric means 5 ± 0, increases to 132 ± 18, $n = 5$, $P < 0.05$, means ± SD, with antimegalin antisera at 1:100 dilution 17 ± 2, at 1:330 dilution 40 ± 3, at 1:1,000 dilution 90 ± 2, $n = 5$, $P < 0.05$). At the same titer, anti-cubilin antiserum had a smaller although significant effect (at 1:100 dilution 48 ± 7, at 1:330 dilution 96 ± 6, at 1:1,000 dilution 116 ± 12, $n = 5$, $P < 0.05$). AT1 antiserum, in contrast, had no effect (at 1:100 dilution 161 ± 14, at 1:330 dilution 149 ± 9, at 1:1,000 dilution 111 ± 19, $n = 5$, $P > 0.05$). Antibodies against megalin and cubilin had an additive effect (at 1:100 dilution 7 ± 4, at 1:330 dilution 23 ± 1, at 1:1,000 dilution 62 ± 5, $n = 5$, $P <$ 0.05 against both anti-megalin and anti-cubilin alone). The effect of antibodies on MT uptake was not observable unless ovalbumin was used to reduce nonspecific effects.

The effect of the synthetic MT-derived peptides (FIG. 1) on MT uptake could be observed when nonspecific binding was carefully excluded. The addition of ovalbumin, which reduces the nonspecific binding of proteins to BN-16 cells (Verroust PJ. et al, 2002 Kidney Int 62: 745–756), unmasked the differential effects of these peptides on the uptake of MT. The greatest effect was produced by peptides containing the KK sequence of the interdomain region of MT: *peptides 2* and *4* and the SCKKSCC overlap peptide reduced binding (FIG. 7*B*) (control 17 ± 1 fluorescence units geometric means ± SD, $n = 5$, MT alone 411 ± 41, *peptide 2* 398 ± 84, $n = 5$, $P > 0.05$, *peptide 4* 349 ± 65, $n = 5$, $P < 0.05$, overlap peptide 280 ± 51, $n =$ 5, $P < 0.05$). When ovalbumin was not included in the incubation medium, however, the differences among the peptides were insignificant. Increasing the ratio of peptide to MT above the 1:20 ratio shown here to look for a larger effect was prevented by peptide solubility.

Uptake was also inhibited by unlabeled MT and recombinant MT domains (FIG. 7*C*). As expected, unlabeled recombinant mouse MT (shown to adopt the native structure; Atrian et al, cited above) competed strongly with the labeled MT (unstained BN cells 4 ± 0 fluorescence units, geometric mean ± SD, $n = 4$, MT alone 129 ±18, $n = 5$, $P < 0.05$ compared with unstained, recombinant MT competition 17 ± 1, $n = 4$, $P < 0.05$). Interestingly, the separated recombinant MT domains did not produce equivalent effects: whereas the recombinant α-domain with an intact interdomain motif inhibited approximately as well as intact MT (9 ± 1, $n = 4$, $P < 0.05$ compared with MT alone), the β-domain with a disrupted interdomain motif had a much smaller effect (68 ± 5, $n = 4$, $P < 0.05$ compared with both MT alone and recombinant MT) (FIG. 7*C*).

In summary, Examples 1-6 provide three lines of evidence that megalin binds MT and that this is the predominant mechanism of uptake of MT and its conjugated heavy metals in the kidney. The hinge region of MT, based around the highly conserved lysine repeat, is one critical peptide sequence for the MT megalin binding interaction. MT fragments and mutants truncating or altering the hinge region prevent megalin-medicated renal uptake of conjugated heavy metals and secondarily diminish or abolish heavy metal renal tubular damage.

While the examples above provide evidence consistent with megalin being the predominant uptake mechanism for MT, we cannot exclude a role for other pathways, especially a role for cubilin. The antibody binding data on both brush-border membrane vesicles and BN-16 cells shows an effect of anti-cubilin antiserum on MT binding and uptake. Megalin is a molecular chaperone for cubilin (Verroust PJ. et al, 2002 Kidney Int 62: 745–756, Verroust PJ and Kozyraki R. 2001 *Curr Opin Nephrol Hypertens* 10: 33–38), so the colocalization studies, not surprisingly, demonstrate colocalization of MT with both cubilin and megalin during the early steps of internalization and uptake. The only data collected against a role for cubilin in MT binding and uptake are the direct studies of molecular interactions using SPR techniques. Although other known ligands of cubilin bound in control studies, partial denaturation of cubilin, which is inevitable during its purification, may mask binding.

Thus, cubilin may also play a role in MT uptake in the proximal tubule of the kidney and other cubilin/megalin-expressing epithelia such as in the placenta.

EXAMPLE 7: NOVEL MUTANT MT-HEAVY METAL COMPLEXES

A. Preparation of recombinant mouse metallothionein I, and α and β subunits.

Recombinant fragments of mouse MT were produced successfully and reproducibly according to the procedures of Atrian et al, cited above. This approach seems more practical than site directed mutagenesis, as most attempts to produce recombinant MT have been characterized by very low yields or by mixtures of several short cleavage fragments of the MT molecule (Sabolic I, et al, 2002. *Am J Physiol Renal Physiol* 283: F1389–F1402; Suzuki-Kurasaki M, et al, 1997 *J Histochem Cytochem* 45: 1493–1501). This problem was resolved by making recombinant MT subunits in *E. coli* using a GST fusion vector followed by thrombin cleavage to release the free MT subunit (see, Atrian et al, cited above). The thrombin cleavage leaves three amino acids, specifically SCM derived from the C-terminus of the GST, on the N-terminus of the product.

It is theorized that the critical binding site on MT is the intradomain SCK-KSCC region (aa 28-34 of SEQ ID NO: 1) with SCK representing the C-terminus end of the β-subunit, and KSCC the N-terminus start of the α subunit. The recombinant α subunit therefore has a conservative GST derived SCM substitution for SCK on its N-terminus, leaving the postulated critical SCKKSCC sequence (aa 28-34 of SEQ ID NO: 1) essentially intact. The recombinant β-subunit starts with SCM- and ends in SCK rendering the postulated critical SCKKSCC (aa 28-34 of SEQ ID NO: 1) disrupted. The full-length recombinant MT has an intact SCKKSCC (aa 28-34 of SEQ ID NO: 1) sequence as well as an addition N-terminal SCM-. Inductively coupled plasma (ICP) analysis of the zinc and platinum content of the recombinant subunits proved them to be at the predicted heavy metal content to within the error of the methods. Protein concentrations were assayed by the Bradford method (Pierce Biotechnology, Rockford, IL).

B. Alkaline phosphatase conjugated metallothionein and subunits.

Metallothionein, recombinant α subunits and recombinant β subunits conjugated to alkaline phosphatase as a reporter of uptake. To conjugate gut isoform alkaline phosphatase one mg of each protein was dialyzed overnight into 0.1M sodium carbonate/bicarbonate buffer at pH 9.8, reacted with activated alkaline phosphatase, according to the method of the AP Labeling kit from Roche Diagnostics (Mannheim Germany). The conjugated proteins were dialyzed overnight into phosphate buffered saline prior to use. Because MT is a very small protein, unreacted dye was removed by dialysis against PBS at pH 7.4 in Slide-A-Lyzer dialysis cassettes having 3500 kD molecular weight cut off (Pierce, cat. no. 66330, Rockford IL), rather than using the columns recommended in the manufacturer's kit.

C. Conjugation of cisplatin into metallothionein and subunits.

To replace all heavy metals in metallothionein with cisplatin, commercial metallothionein I (Sigma Chemical, St Louis MO) was combined in a ratio of 5:3 metallothionein to cisplatin by weight in 10mM HEPES buffer, and incubated at 37°C for 48 hours. The product was dialysed against PBS or carbonate buffer as appropriate for toxicity and uptake experiments.

To remove cadmium in commercial metallothionein and replace with zinc, 5mg of mouse metallothionein I was put into solution in 10mM HEPES buffered to pH 3.0 degassed with dry nitrogen to strip all heavy metals. Then zinc was added by dialysis against a buffer containing 2mM beta-mercaptoethanol, and 1mM zinc chloride in 10mM HEPES pH 7.4. All metallothionein reagents had their platinum contents confirmed by inductively coupled plasma.

EXAMPLE 8: METALLOTHIONEIN-CISPLATIN COMPLEXES, THEIR NEPHROTOXICITY AND TOXICITY RELATIVE TO CISPLATIN ALONE.

CD-1 mice received a single intraperitoneal injection of 20mg/kg cisplatin either alone or conjugated to full length or recombinant metallothionein subunits prepared as in Example 7. The mice were sacrificed 48 hours later and blood collected for creatinine analysis as an index of renal function. Aliquots of kidney and liver tissues and a set aside aliquot of the blood were harvested for subsequent analysis of

platinum content by inductively coupled plasma. The serum creatinine in mg/dl was assayed using a plate based colorimetric assay (Cayman Chemical, Ann Arbor MI) on blood drawn 48 hours after cisplatin treatment of mice.

The results are demonstrated in FIG. 7. The creatinine values were 0.055±0.022 in the group of mice treated with phosphate buffered saline (PBS) vehicle (mean ± standard error, n=5); following cisplatin treatment (cis) creatinine increased significantly to 0.105 ± 0.056 mg/dl; metallothionein alone (ZnMT) resulted in creatinine values of 0.068 ± 0.028; metallothionein carrying cisplatin (MT-cis) 0.083 ± 0.053; alpha subunit carrying cisplatin (alpha cis) 0.069 ± 0.030; and beta subunit carrying cisplatin (beta cis) 0.060 ± 0.023. The groups significantly different from phosphate buffered control at $p<0.05$ level by one-way ANOVA and Tukey's post-hoc comparison are the cisplatin alone and MT-cis groups. The beta cisplatin group is significantly less than the cisplatin alone group, demonstrating renal protection with this reagent.

EXAMPLE 8: CYTOTOXICITY AND ANTI-TUMOR EFFICACY *IN VITRO* OF METALLOTHIONEIN-CISPLATIN COMPLEXES.

The cytotoxicity and anti-tumor efficacy of the MT-Cis complexes of Example 7 were determined by testing for uptake of metallothionein and its fragments into lung cancer (small cell carcinoma), invasive transitional cell bladder cancer (J82) cells and ovarian carcinoma cells (OVCAR-3), and conducting an assay of induction of apoptosis as evidenced by caspase 3 levels.

A. Culture of cancer cell lines.

J82 cells, a bladder transitional cell carcinoma cell line, was cultured in EMEM alpha medium with 10% fetal calf serum and cisporofloxacin. DMS53, small cell lung carcinoma of the lung was cultured in Waymouth's MB 752/1 culture media with 10% fetal calf serum and ciprofloxacin. Cells were grown to near confluence in T75 flasks and then split into 96 well plates for study. OVCAR_3 cells were cultured in DMEM with 10% fetal calf serum and pen/strep.

B. Statistics.

Data are expressed as mean ± standard error of the mean throughout these examples. Statistical analysis was performed by analysis of variance and Tukey's post hoc comparison.

C. Metallothionein uptake by cells analyzed by absorption spectrometry in lung cancer cells (small cell carcinoma).

Uptake experiments using alkaline phosphatase conjugated to metallothionein and its recombinant subunits were performed with confluent monolayers cultured in 96-well plates. In preliminary experiments, metallothionein uptake was linear for at least 4 hours and exhibited dose-dependent saturation. Experiments are performed as follows. The confluent monolayers are washed with serum-free media and allowed to equilibrate at for two hours at 37 °C. The cells are then incubated with 10 μM labeled metallothionein for 4.0 hours at 37 °C. The cells are washed several times with PBS, acid-washed to release membrane-bound proteins, and washed several more times with PBS.

Alkaline phosphatase activity is assayed in the wells by addition of alkaline phosphatase yellow (pNPP) liquid substrate system (Sigma Chemical Company St Louis, MO.) and incubation. Alkaline phosphatase is then analyzed immediately on a plate reader using absorbance as the endpoint.

As illustrated by FIG. 8, the absorbance of the alkaline phosphatase conjugated to the metallothionein and its subunits following uptake into lung small cell carcinoma cells for 4 hours was 0.031±0.005 in phosphate buffered saline vehicle (mean ± standard error, n=6). Following 0.5mM cisplatin treatment for 4 hours, this increased significantly to 0.068 ± 0.015. Metallothionein alone was 0.25 ± 0.04; and metallothionein carrying 0.5mM cisplatin was 0.46 ± 0.03. The MT-α subunit carrying was 0.5mM cisplatin 0.31 ± 0.06; and MT-β subunit carrying 0.5 mM cisplatin was 0.37 ± 0.06.

All other groups are significantly different from phosphate buffered saline control at $p<0.05$ level by one-way ANOVA and Tukey's post-hoc comparison in all groups compared to phosphate buffered saline other than the cisplatin group.

D. Caspase-3 activation in cell treated with cisplatin conjugated to metallothionein and its subunits in lung cancer cells.

To determine metallothionein and its subunits induce caspase-3 activation, commonly associated with induction of cell death by apoptosis, cells were treated as in the uptake protocol above and the caspase-3 therein was assayed.

CaspACE™ FITC-VAD-FMK *in situ* marker (Promega, Madison WI) is a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). The fluoroscein isothiocyanate (FITC) group has been substituted for the carbobenzoxy (Z) N-terminal blocking group to create the fluorescent apoptosis marker. This structure allows delivery of the inhibitor into the cell where it irreversibly binds to activated caspases. The FITC label allows for a single-reagent addition to assay activated caspase-3 activity *in situ*.

As shown in FIG. 9, the absorbance of the activated caspase-3 specific probe was 0.007849±0.00483 in phosphate buffered saline vehicle (mean ± standard error, n=6). Following 0.5mM cisplatin treatment for 4 hours, this result increased significantly to 0.012656 ± 0.008571, $p<0.05$. Metallothionein alone was 0.036802 ± 0.015034; metallothionein carrying 0.5mM cisplatin was 0.015741 ± 0.011293; MT-α subunit carrying 0.5mM cisplatin was 0.027545 ± 0.023401; and MT-β subunit carrying 0.5 mM cisplatin was 0.019419 ± 0.011565. All other groups were significantly different from phosphate buffered saline control at $p<0.05$ level by one-way ANOVA and Tukey's post-hoc comparison.

These results demonstrate that cisplatin is equally or more effective in inducing apoptosis in small cell carcinoma cells when conjugated to metallothionein fragments compared to treatment with unconjugated cisplatin.

E. Metallothionein uptake by cells analyzed by absorption spectrometry in bladder cancer cells (J82 transitional cell carcinoma)

Uptake experiments using alkaline phosphatase conjugated to metallothionein and its recombinant subunits, were performed with confluent monolayers cultured in 96-well plates as for the other cell lines. As shown in FIG. 10, the absorbance of the alkaline phosphatase conjugated to the metallothionein and its subunits following uptake into lung small cell carcinoma cells for 4 hours was 0.051±0.006 in phosphate

buffered saline vehicle (mean ± standard error, n=6). Following 0.5mM cisplatin treatment for 4 hours, this changed to 0.082 ± 0.016; metallothionein alone 0.27 ± 0.04; metallothionein carrying 0.5mM cisplatin 0.48 ± 0.03; alpha subunit carrying 0.5mM cisplatin 0.33 ± 0.04; and beta subunit carrying 0.5 mM cisplatin 0.39 ± 0.06.

All other groups are significantly different from phosphate buffered saline control at $p<0.05$ level by one-way ANOVA and Tukey's post-hoc comparison in all groups compared to phosphate buffered saline other than the cisplatin group.

F. Caspase-3 activation in cell treated with cisplatin conjugated to metallothionein and its subunits in bladder cancer cells.

To determine whether metallothionein and its subunits induce caspase-3 activation, commonly associated with induction of cell death by apoptosis, cells treated as in the uptake protocol above had caspase-3 assayed. As illustrated in FIG. 11, the absorbance of the activated caspase-3 specific probe was 0.017± 0.007in phosphate buffered saline vehicle (mean ± standard error, n=6). Following 0.5mM cisplatin treatment for 4 hours, this increased significantly to 0.038± 0.016, $p<0.05$; metallothionein alone 0.019± 0.008; metallothionein carrying 0.5mM cisplatin 0.042± 0.017; alpha subunit carrying 0.5mM cisplatin 0.053±0.021; and beta subunit carrying 0.5 mM cisplatin 0.043±0.018.

All other groups are significantly different from phosphate buffered saline control at $p<0.05$ level by one-way ANOVA and Tukey's post-hoc comparison except metallothionein alone.

In a second performance of the bladder cell studies, J82 transitional cell carcinoma cells were grown in 96 well plates. Following growth to near confluence the cells were changed to serum free medium, and 0.5 mM cisplatin in serum free medium added either alone or conjugated to the beta subunit of metallothionein. The beta subunit of metallothionein was prepared recombinantly, and cisplatin was conjugated by incubation at 37°C for 48 hours. Residual excess cisplatin was removed by overnight dialysis against phosphate buffered saline. After 4, 8 or 22 hours of incubation in cisplatin, the cells were washed with phosphate buffered saline and activated caspase –3 labeled by addition of a fluorescent cell permeable peptide which binds specifically to caspase-3. After 30 minutes labeling with the caspase-3 peptide,

cells were again washed with phosphate buffered saline, trypsinized off the plates and caspase-3 activation assayed by flow cytometry using fluorescence of the fluorescent peptide as a reporter. The results in FIG. 14 show that cisplatin administered conjugated to the beta subunit of metallothionein induces at least as much activation of caspase-3, and hence cell death by apoptosis as cisplatin alone.

G. Ovarian Carcinoma (OVCAR-3) cells take up MT

To determine if OVCAR3 cells take up MT, the cells were incubated in fluorescently labeled MT. OVCAR3 cells were grown to confluence in 96 well plates, and serum starved before exposure to 40μM Cy3-fluorescently conjugated MT for 1,2 and 4 hours. Cells were washed, surface binding released with acid, and trypsinized for flow cytometry analysis of MT uptake.

Compared to unstained cells (0±0) fluorescent units, the MT was taken up significantly by 1 hour (10±3fluorescent units, n=4, $p<0.05$ compared to unstained), 2 hours (13±3fluorescent units, n=4, $p<0.05$ compared to unstained), or 4 hours 1 hour (16±2 fluorescent units, n=4, $p<0.05$ compared to unstained) as illustrated in FIG. 12.

H. Cisplatin and MT-cisplatin kill OVCAR cells.

To determine if our OVCAR3 cells are a good model for cisplatin efficacy, the cells were exposed to cisplatin. As in the protocols above the confluence cells in 96 well plates were serum starved for 2 hours, prior to addition of cisplatin, MT complexed cisplatin, or the MT carrier alone. Cell death was assayed by trypsinizing the cells off the plates, staining with propridium iodide and assaying loss of cell membrane integrity by flow cytometry.

Preliminary studies on dose and time course showed 250μM for 24 hours results in significant cell death, and these parameters were adopted for these comparison studies. As expected, cisplatin kills OVCAR3 cells (untreated OVCAR3 cells 20±5 fluorescence units, geometric mean ± standard deviation n=4, cisplatin alone 53±2, n=4, $p<0.05$ compared to untreated, cisplatin complexed to MT 41±5, n=4, $p<0.05$, and MT alone 20±6, not significant). The results are demonstrated in FIG. 13 and demonstrate that MT is taken up into OVCAR3 cells and that MT complexed cisplatin has almost the same efficacy in cell killing as free cisplatin.

In summary, the above Examples 7 and 8 demonstrate that complexes of the beta subunit of metallothionein and cisplatin are significantly less nephrotoxic that cisplatin alone. Metallothionein-cisplatin complexes retain cytotoxic anti-tumor efficacy *in vitro*.

All documents, including Klassen *et al*, *Am. J. Phsiol. Renal. Physiol, 287*:F393-403 (May 4, **2004**), the priority document, and public databases cited within this specification are incorporated herein by reference.